
QUAECUMQUE VERA

THE UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR .... Peter L. Aster ....

TITLE OF THESIS .... The Influence of Temperature and Seasonal Acclimatization on Glucose-6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase from Three Teleost Species ....

DEGREE FOR WHICH THESIS WAS PRESENTED .... Master of Science ....

YEAR THIS DEGREE GRANTED .... 1976 ....

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

THE UNIVERSITY OF ALBERTA

THE INFLUENCE OF TEMPERATURE AND SEASONAL ACCLIMATIZATION ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND 6-PHOSPHOGLUCONATE DEHYDROGENASE FROM THREE TELEOST SPECIES

by

© PETER L. ASTER

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF ZOOLOGY

EDMONTON, ALBERTA

FALL, 1976

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "The influence of temperature and seasonal acclimatization on glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase from three teleost species" submitted by Peter L. Aster, in partial fulfillment of the requirements for the degree of Master of Science.

ABSTRACT

1. Goldfish (*Carassius auratus*) and white sucker (*Catostomus commersoni*) liver glucose-6-phosphate dehydrogenase (G6PD) exhibited 8 to 30 times the activity of northern pike (*Esox lucius*) G6PD assayed under similar conditions. Liver 6-phosphogluconate dehydrogenase (6PGD) activity of goldfish and white suckers was at least twice that found in northern pike.

2. The activity per gram liver of G6PD from northern pike was slightly (20%) higher in winter than in summer caught fish, while sucker liver G6PD activity showed no changes in winter vs. summer caught fish. No consistent temperature acclimation-dependent differences in G6PD activity of goldfish liver were observed.

3. G6PD activity from all three species was strongly inhibited by NADPH (Ki ca. 18-46 μM).

4. No differences in Michaelis constants (Km) of G6PD and 6PGD were observed in winter vs. summer caught pike and suckers, or in cold- (2 C) vs. warm- (20 C) acclimated goldfish, suggesting that no isozymic changes occurred in these enzymes due to temperature acclimation or season in the three species of fish examined.

5. The Km(G6P) of goldfish and white sucker G6PD exhibited extensive positive thermal modulation (ca. 100 to 38-50 μM) between 22 C and 2 C, while at temperatures below 2 C, negative thermal modulation occurred in the white sucker enzyme. The lowest Km(G6P) for all three species was similar (38-50 μM) and occurred at low (2-3 C) temperatures. The Km(NADP) for sucker and pike G6PD was 14-15 μM and 20 μM,

respectively, and was unmodified by temperature, indicating that temperature compensation does not occur by significant reductions in the Km values of the rate-limiting factor, $NADP^+$.

6. The Km(6PG) of 6PGD was insensitive to temperature modulation over a 20 C range, and exhibited similar values (ca. 30-40 μM) in all three species.

7. Arrhenius plots of liver G6PD from all three species were non-linear between 22 C and 2 C, with higher energy of activation values at lower temperatures (5-2 C for goldfish and suckers; 12-2 C for pike). Arrhenius plots for sucker and pike liver 6PGD were linear over a 20 C temperature range, but goldfish liver 6PGD exhibited a non-linear Arrhenius plot similar to G6PD.

8. Liver tissue showed the highest pentose phosphate pathway dehydrogenase (G6PD, 6PGD) activity, as well as high malic enzyme, $NADP^+$-dependent isocitrate dehydrogenase, and *alpha*-glycerophosphate dehydrogenase activity, suggesting that the liver is the primary organ of *de novo* lipogenesis. Pike liver, however, had no detectable malic enzyme activity.

9. Muscle tissue from all three species had high $NADP^+$-dependent isocitrate dehydrogenase activity, and low G6PD, 6PGD, and malic enzyme activity.

10. An examination of the temperature-dependent properties of the pentose phosphate pathway dehydrogenases suggests that, on an immediate or seasonal basis, only a minor amount of rate compensation for temperature takes place. Previously reported increases in pentose phosphate pathway utilization in carbohydrate catabolism during cold-

acclimation do not appear to have been the result of any temperature-dependent properties of the pathway dehydrogenase enzymes.

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to the following:

Dr. W. C. Mackay for providing enthusiastic supervision and encouragement;

Dr. D. D. Beatty and Dr. L. P. Milligan for their critical review of the manuscript;

Brian Medford for the many profitable discussions about northern pike and cuttlefish, and for invaluable assistance in the procurement of animals for this study;

the fellow graduate students who provided aid and moral support;

the biologists from other universities who provided stimulating ideas and constructive criticism; and

Susan Enders for her assistance in the preparation of the manuscript.

ABBREVIATIONS

Enzymes

G6PD = glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:$NADP^+$ oxidoreductase, E.C. 1.1.1.49)

6PGD = 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:$NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.44)

ME = malic enzyme (L-malate:$NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.40)

ICD = $NADP^+$-dependent isocitrate dehydrogenase (*threo*-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating), E.C. 1.1.1.42)

αGPD = *alpha*-glycerophosphate dehydrogenase (L-glycerol-3-phosphate:NAD^+ oxidoreductase, E.C. 1.1.1.8)

Substrates and Coenzymes, etc.

G6P = glucose-6-phosphate

6PG = 6-phosphogluconate

αGP = *alpha*-glycerophosphate

NAD^+/NADH = oxidized/reduced forms of nicotinamide-adenine dinucleotide

$NADP^+$/NADPH = oxidized/reduced forms of nicotinamide-adenine dinucleotide phosphate

EDTA = ethylenediaminetetraacetic acid

PP pathway = pentose phosphate pathway (hexose monophosphate shunt)

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIX TABLES

Page

APPENDIX I

APPENDIX II

APPENDIX IV

APPENDIX V

APPENDIX VI

LIST OF APPENDIX FIGURES

Page

APPENDIX I

APPENDIX III

APPENDIX IV

Page

APPENDIX IV

APPENDIX VII

INTRODUCTION

Much of the interest in temperature acclimation by fish physiologists stems from the work of Hochachka and Hayes (1962), in which it was shown that, in brook trout (*Salvelinus fontinalis*), there was a "metabolic reorganization" during cold acclimation, during which the utilization of the pentose phosphate pathway (PP pathway) in carbohydrate catabolism was proportionally increased. In addition, cold-acclimated fish showed a proportional increase in the incorporation of ^{14}C-acetate into lipid. They concluded that cold acclimation favored extramitochondrial pathways, particularly those related to lipogenesis. Similar conclusions were reached by Ekberg (1958) and Kanungo and Prosser (1959) based on the effects of metabolic inhibitors on the respiration of tissues from warm- and cold-acclimated goldfish, *Carassius auratus*.

The above studies suggested that proportionally increased use of the PP pathway might be a general phenomenon in cold-acclimated fish, with a common molecular basis. The two enzymes of the oxidative portion of the pentose phosphate pathway, glucose-6-phosphate dehydrogenase (G6PD) and 6-phosphogluconate dehydrogenase (6PGD), may have evolved adaptive properties which could account for the above changes during cold acclimation. The former enzyme controls the entry of G6P into, and flow through the pathway (Greenbaum et al., 1971). The latter enzyme (6PGD) increased in activity in crucian carp during cold acclimation (Ekberg, 1962).

The proportionally increased use of the PP pathway during cold acclimation is presumably to supply reducing equivalents (NADPH) for increased lipogenesis. In mammalian tissues, the pentose phosphate pathway accounts for 60% or more of the NADPH required for lipogenesis (Flatt and Ball, 1964), and the activity of the pathway dehydrogenases (Pande et al., 1964) and carbon flow through the pathway (Greenbaum et al., 1971) have been positively correlated with the rates of *de novo* lipogenesis. Evidence for increased lipogenesis in cold acclimation comes primarily from the work of Knipprath and Mead (1968) on goldfish and the work of Dean (1969) on rainbow trout, *Salmo gairdneri*. Unfortunately, our knowledge of *de novo* lipogenesis in fish, particularly its enzymology, consists primarily of the assumption that it is similar to mammalian lipogenesis.

A primary reason for increased lipogenesis during winter acclimatization is undoubtedly the production of new membrane lipids which are better suited for the maintenance of membrane structure and function at low temperatures (Knipprath and Mead, 1968). However, increased lipid synthesis for energy storage (see reviews by Love, 1970; Shul'man, 1974) and/or gonad development must be considered, particularly in naturally acclimatized animals. Das (1967) has shown that, during cold acclimation, there is increased RNA synthesis in goldfish liver and muscle, and the PP pathway may also be important in supplying pentose sugars for this process. Furthermore, changes in metabolic organization, such as increased lipogenesis, which appear to be brought about strictly by cold acclimatization, may not be clearly separable from

those changes caused by reproductive cycles.

The molecular mechanisms involved in a shift towards a proportional increase in PP pathway participation in carbohydrate catabolism during cold acclimation have not been adequately studied, although much has been learned in recent years about the molecular mechanisms of temperature compensation and acclimation (Hochachka, 1967; Hochachka and Somero, 1971, 1973; Hazel and Prosser, 1974). Most of the work, however, has concentrated on laboratory acclimated animals, rather than animals acclimatized in their natural environment. It has been demonstrated (see reviews cited above) that poikilotherms often use enzymic mechanisms to compensate for changes in the kinetic energy of their environment. These mechanisms of temperature compensation may always be present, thus allowing for instantaneous as well as seasonal compensation, or they may develop only after periods of acclimation or acclimatization. Even if molecular mechanisms for rate compensation are continually present, they may only be utilized at certain times of year in the animal's natural habitat. The enzymic mechanisms or strategies used for this temperature compensation may be classified as: modulation, in which the reaction rates of enzymes already present in the cell are changed by temperature, metabolites, pH, ions, etc.; quantitative, in which the concentration or total quantity of pre-existing enzyme types is increased; and qualitative, in which the types of enzyme in the cell are changed (Hochachka and Somero, 1973).

In 1973, Hochachka and Hochachka showed that cold-acclimated mullet fish (*Mugil cephalus*), a subtropical species, produce an isozyme of glucose-6-phosphate dehydrogenase not present in significant amounts in

warm-acclimated fish (qualitative strategy). This G6PD isozyme showed temperature-dependent substrate, coenzyme, and inhibitor kinetics (modulation strategy) which might account for or facilitate proportionally increased PP pathway utilization in cold-acclimated fish. However, such isozymic changes with cold acclimation are unusual in a non-salmonid species, such as the mullet (Somero, 1975a). Furthermore, if proportionally increased utilization of the pentose phosphate pathway is a necessary adjustment for survival in cold-acclimated fish, then one would expect the enzymic mechanisms causing this shift to be best developed in those fish which must seasonally adjust to cold temperatures in nature, such as north temperate freshwater species, rather than in laboratory acclimated, subtropical marine species.

Therefore, the present study examines the temperature-dependent activities and kinetics of the enzymes of the oxidative portion of the pentose phosphate pathway in tissues from natural, north temperate populations of white suckers (*Catostomus commersoni*) and northern pike (*Esox lucius*), and from a laboratory population of goldfish (*Carassius auratus*). Northern pike and white suckers were chosen because of the distinct differences in their life styles, as well as their availability at different times of the year. The northern pike is a predator, feeding primarily on other fish, while the sucker feeds along the bottom on aquatic invertebrates, etc. (Scott and Crossman, 1973). The goldfish were chosen as a laboratory species because of the extensive literature on cold acclimation which exists for this animal.

Liver PP pathway enzymes were the most thoroughly studied, since the liver appeared to be the primary organ of lipid storage (Tashima and

Cahill, 1965) and of *de novo* lipogenesis (Tashima and Cahill, 1965; Kluytmans and Zandee, 1974), and the G6PD reaction was particularly emphasized, since it controls the pathway (at least in rat liver, Greenbaum et al., 1971). However, other tissues and enzymes were examined briefly to confirm that the liver was important in lipid metabolism, and to more completely assess the sources of reducing equivalents for lipogenesis. Lipid content in whole goldfish and white sucker tissues and glycogen content in goldfish tissues were also determined to complement the enzyme studies.

The following questions were asked:

1. What are the strategies or mechanisms used, if any, to compensate for changes in PP pathway dehydrogenase activity brought about by the 22 C range of environmental temperatures to which these fish are exposed annually in their natural habitat? Do the temperature-dependent properties of the enzymes studied suggest that there is any "seasonal metabolic reorganization" which would result in a relative increase in carbon flow through the pentose phosphate pathway, and which would be indicative of proportionally increased rates of lipogenesis?

2. What are the patterns of lipogenic and NADPH generating activity in various tissues of the different species of fish? What do these patterns suggest about the function of G6PD, 6PGD, malic enzyme, isocitrate dehydrogenase, and *alpha*-glycerophosphate dehydrogenase in these tissues, and the potential significance of each tissue to *de novo* lipogenesis?

MATERIALS AND METHODS

A. ANIMALS

Common goldfish (*Carassius auratus* Linnaeus) weighing from 6 to 20 g were purchased from the Grassyfork Fisheries Company of Martinsville, Indiana. Upon arrival at the University of Alberta, fish were maintained in 150 l tanks at 20 C (±3 C) for at least 4 weeks prior to transfer to colder temperatures. Animals to be cold-acclimated were transferred to a cold room (8-12 C) for 2 to 4 weeks and then moved to various refrigerated rooms (1-4 C, depending on the room), with a photoperiod of 2-6L:22-18D. The photoperiod for the warm-acclimated fish was approximately 12L:12D in winter and 14L:10D in summer.

Cold-acclimated animals were kept in groups of 5 to 16 in 19 or 54 l tanks. Both warm- and cold-acclimated fish were maintained in demineralized water containing 0.1% Seven Seas Marine Mix Salts (Utility Chemical Co., Paterson, N.J.) and 0.1% Fish Grade Tris Buffer pH 7.4 (Sigma Chem. Co., St. Louis, Mo.). The pH of the buffered water increased at lower temperatures, and was approximately pH 8.0 at 2 C. The bottom of each tank was covered with a layer of activated charcoal to adsorb noxious materials.

Goldfish were fed with SeRa Goldy (SeRa Co., West Germany) or Tetramin Staple Food (TetraWerke, West Germany). For warm-acclimated fish, minimum daily food levels were approximately 1% of the fish biomass in each tank. Fish held at lower temperatures were fed one-half of this amount or less, but always in excess, as indicated by food particles on

the bottom of the tank the following day. In some experiments (see Table 8), cold-acclimated fish were not fed.

Specimens of white sucker (*Catostomus commersoni* Lacépède) and northern pike (*Esox lucius* Linnaeus), weighing approximately 1,000 g (range 738-1,387), were collected in March (winter-caught fish) and July (summer-caught fish) by gill netting from Lac Ste Anne, a shallow eutrophic lake, located approximately 80 km northwest of Edmonton, Alberta. Only fish that appeared freshly caught were used. A few specimens were collected in late April (1974) and early May (1975) during their spawning migration in a temporary stream draining into Lac Ste Anne.

When the lake was ice-covered in March, the water temperature was 0.6 C (0-1 C, depending on the depth). In April (1974), water temperature of the temporary stream was 3-4 C, and in May (1975), it was 10-11 C. The water temperatures of Lac Ste Anne range from 16.5-22.0 C in July (Medford, 1976; Mackay and Beatty, 1968). Oxygen levels in Lac Ste Anne range from about 9.5 ppm in July to 2.6 ppm in March (Langer, 1974), and are probably not stressful to either species.

B. TISSUE PREPARATION

Goldfish were stunned by a blow on the head, decapitated, and the liver or muscle (white epaxial muscle) removed and weighed immediately. Tissues from 3 to 5 goldfish of either sex were pooled and treated as described below for sucker and pike tissues.

Goldfish liver is a rather diffuse organ, consisting of a large anterior mass which is associated with the gall bladder, and several

posterior sections lying along the digestive tract. Fat cells may be integrated with the liver, especially posteriorly. In addition, the pancreas is not easily distinguished macroscopically from the liver. Hence the liver preparations used in this study not only included hepatic tissue *per se*, but also adipocytes and pancreatic tissue. Some effort was made to standardize the liver preparations by taking 75-85% by weight of the sample from the distinct anterior hepatic tissue mass.

For sucker and pike preparations, animals were removed from the net, killed by a blow on the head, and placed on ice. Tissues were removed within one hour and placed on dry ice for transport back to the laboratory.

Pike liver samples consisted of the most posterior 3 cm portion. Sucker liver was sampled in a manner similar to goldfish liver. Muscle samples (white epaxial muscle) were removed from the 5 cm area approximately 3-8 cm posterior to the head.

Immediately upon return to the laboratory, tissues from 2 to 5 white suckers and northern pike were pooled. For sucker and pike homogenates, only tissues from male animals were used. The samples were diluted with 3 to 10 volumes of ice-cold 5 mM EDTA solution, then homogenized for 2 minutes with a motor-driven teflon-glass homogenizer (Thomas & Co., Phila.), which was placed in an ice bath. The homogenate was filtered through a layer of cheesecloth and centrifuged; 10,000 x g at 0 C for a minimum of 20 minutes in a Sorvall RC-2 refrigerated centrifuge (Sorvall & Co., Norwalk, Conn.). The supernatant fraction was then recentrifuged at 18,000 x g for a minimum of 45 minutes; and the supernatant fluid from this centrifugation was diluted, if necessary,

and stored in 2-3 ml batches at -20 to -30 C until used. No further purification of the enzyme was attempted. This preparation will henceforth be called the homogenate.

In retrospect, homogenization of the tissues with hypotonic 5mM EDTA solution was a poor choice, since it probably resulted in the rupture of cell organelles and consequent release of organelle enzymes. Some of these enzymes, such as NADPH-cytochrome *c* reductase and NADPH oxidase utilize NADPH and thus may interfere with the dehydrogenase assays (see Appendix VII). Furthermore, catheptic enzymes may have been released from the ruptured lysosomes, resulting in protein denaturation. Nonetheless, it should be recognized that homogenization media which provide an isotonic or buffered environment, while superior to hypotonic solutions, do have disadvantages as well. For example, both sucrose (Gerhardt and Beevers, 1968) and Tris-HCl buffer (Gellert et al., 1959) have been reported to interfere with some of the modifications of the Lowry protein determination (Lowry et al., 1951), although sucrose does not appear to interfere appreciably with the unmodified Lowry method used in this study (Bonitati et al., 1969). Homogenization with isotonic KCl would present difficulties in assessing the effects of ionic strength on enzyme activity (Appendix I).

C. ENZYME ASSAYS

Enzyme activity in the homogenate was assayed by recording the reduction of $NADP^+$ (or NAD^+) as shown by the increase in absorbance at 340 nm using a Hitachi-Perkin Elmer 139 UV-vis spectrophotometer. To achieve temperature control, the cuvette chamber was attached to a Lauda

K-2/R Temperature Controller filled with ethylene glycol. The temperature of the solution in the cuvette was measured before and immediately after each assay with a YSI telethermometer probe (Yellow Springs Instrument Co., Ohio), and recorded to the nearest 0.1 C, interpolating when necessary. For each determination of kinetic constants, the mean temperature of all the individual cuvette assay temperatures was considered to be the true assay temperature for the constants, and is reported as such in all later figures and tables. Individual assays which were not within ±0.3 C of the mean temperature were discarded.

Preliminary work with the dehydrogenases indicated that, at the homogenate concentrations used, the reaction rates were linear for a minimum of 2.0 minutes (Fig. 1, Appendix VII). Proportionality was observed for all enzymes, using a two- or threefold range of homogenate concentrations (Fig. 2, Appendix VII). The pH optima for the goldfish dehydrogenases were determined at 22 C with Tris-HCl buffer (100 mM) (Fig. 1A, B, Appendix I). The pH values of the buffer solutions were determined to 0.005 pH units with a Beckman Research pH meter which was standardized each time it was used.

Far all enzyme assays, a 3.0 ml volume was used in a cuvette with a 1 cm light path. An extinction coefficient of 6.22 x 10^3 liter $mole^{-1}$ cm^{-1} was used for all calculations (Kornberg and Horecker, 1955).

All organic reagents used in the enzyme assays were purchased from Sigma Chemical Co., St. Louis, and were of the highest purity available. All substances used were sodium salts. Other chemicals were of reagent grade, and were purchased from Baker, Mallinckrodt, and Fisher.

The glucose-6-phosphate dehydrogenase (G6PD) assay mixture

routinely employed in this study contained (final concentrations): Tris-HCl buffer (100 mM, pH 8.0, temperature adjusted); $MgCl_2$ (10 mM); a saturating concentration of $NADP^+$ (0.4 mM); and varied concentrations of homogenate and D-glucose-6-phosphate (G6P) (Glock and McLean, 1953; Kornberg and Horecker, 1955; see Appendix II). $NADP^+$ concentrations were varied when the Km($NADP^+$) and Ki(NADPH) were determined. The 6-phosphogluconate dehydrogenase (6PGD) assay mixture was identical except for a saturating $NADP^+$ concentration of 0.3 mM, and the substitution of 6-phosphogluconate (6PG) as substrate instead of glucose-6-phosphate (Horecker and Smyrniotis, 1955). The assay was run for a minimum of 2.0 minutes. Activity was negligible using the goldfish homogenate, when glucose (100 mM) was used as the substrate, or when NAD^+ (0.5 mM) was used as the cofactor.

Other dehydrogenase activities were measured using the following assay mixtures:

The malic enzyme assay mixture contained (final concentrations): Tris-HCl buffer (100 mM, pH 8.0); malate (0.1 mM); $NADP^+$ (0.2 mM); $MgCl_2$ (3 mM); and 0.05 to 0.3 ml of homogenate. This is, with slight modification, the procedure of Wise and Ball (1964).

NADP-dependent isocitrate dehydrogenase ($NADP^+$-ICD) was assayed using the procedure of Moon and Hochachka (1971). The cuvette contained (final concentrations): Tris-HCl buffer (100 mM, pH 8.0); $NADP^+$ (0.2 mM); $MgCl_2$ (1 mM); DL-isocitrate (0.1 mM, which is 0.05 mM of the active *threo*-D_s form); and 0.01 to 0.2 ml of homogenate.

Alpha-glycerophosphate dehydrogenase (αGPD) was assayed in the direction of NAD^+ reduction and dihydroxyacetone production, although

this is not always the physiological direction. The procedure followed was that of Bewley et al. (1974). The assay mixture contained (final concentrations): glycine-NaOH buffer (100 mM, pH 9.5); NAD^+ (5 mM); DL-*alpha*-glycerophosphate (15 mM); and 0.05 to 0.2 ml homogenate.

In all of the above enzyme assays, endogenous $NADP^+$ (or NAD^+) reduction, when significant, was allowed to proceed until a constant, negligible rate was recorded, usually within 3 to 5 minutes, then the substrate was added. This was necessary only when the homogenate was freshly prepared, and not previously frozen. $NADP^+$ was added last when it was established that this caused no detectable error (Fig. 1, Appendix VII). NADPH oxidizing reactions were undetectable in goldfish and sucker homogenates, but were detectable, at rates not exceeding 0.001 μmol per minute, in very concentrated pike liver homogenates (Fig. 1, Appendix VII). Appropriate blank cuvettes were used when necessary.

The Km values in this study are the substrate concentrations at which the velocity of the enzyme reaction is one-half of its extrapolated maximum velocity (Vmax). The Km can also be termed the apparent Km, or the reciprocal of the apparent enzyme-substrate affinity (Hochachka and Somero, 1973), to distinguish it from the Ks, the enzyme-substrate dissociation constant (Dixon and Webb, 1964). Calculation of the Km and Vmax is discussed in Appendix VII.

D. PROTEIN

The protein content of the tissue homogenates was determined with Folin-Ciocalteu reagent by the method of Lowry et al. (1951). Absorbance was measured at 500 nm. Bovine serum albumin was used as a standard.

RESULTS

A. THE EFFECT OF TEMPERATURE ON SUBSTRATE-DEPENDENT G6PD KINETICS

All of the glucose-6-phosphate dehydrogenases showed typical Michaelis-Menten hyperbolas when velocity was plotted against varied substrate concentrations. The enzymes were saturated at about 0.2 to 0.5 mM of substrate.

Michaelis constants, Km(G6P) for G6PD, estimated from the linear, non-saturating, non-activating portions of double-reciprocal plots (Appendix III), are recorded as a function of temperature in Table 1 and Figures 1A, B. The temperatures are representative of those encountered by white suckers (*Catostomus*) and northern pike (*Esox*) in the yearly cycle at Lac Ste Anne, and by goldfish (*Carassius*) in the north temperate area of North America.

It was found that the only differences in the Km(G6P) values were interspecific or due to the different assay temperatures (Table 1). There were no differences in the Km values of G6PD enzymes from animals caught at two different times of year or from animals acclimated in the laboratory to two different temperatures (Table 1), suggesting that no qualitative changes in G6PD occur due to season or temperature acclimation.

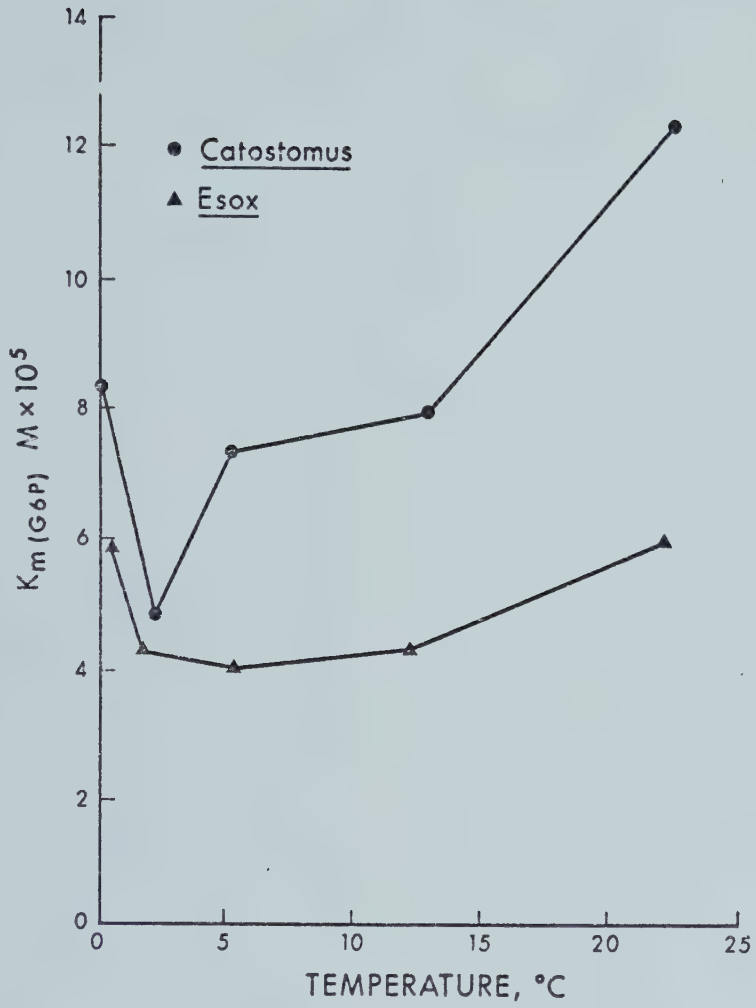
Goldfish G6PD showed a substantial decrease in Km(G6P) between 22 C and 5 C, that is, there was an increase in the apparent enzyme-substrate (E-S) affinity as the temperature decreased (Fig. 1A). This

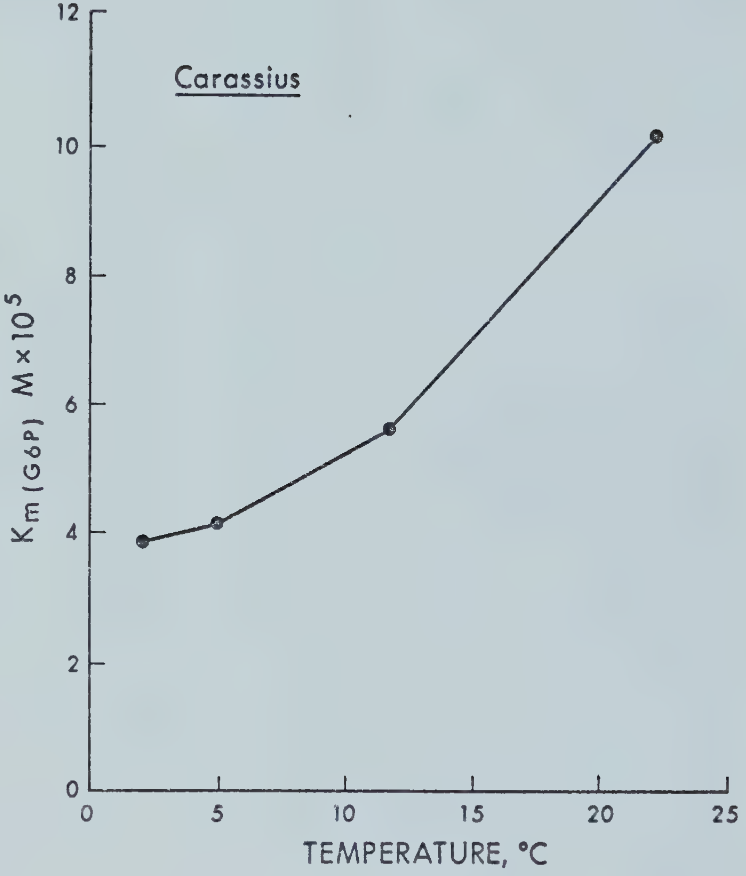
Table 1. Michaelis constants, Km(G6P), for goldfish (*Carassius*), white sucker (*Catostomus*), and northern pike (*Esox*) liver G6PD from differently acclimated or acclimatized groups of fish. Assay temperatures are within ±1 C. Km values were determined from double-reciprocal plots (Appendix III), and are expressed as micromoles of substrate per liter. The livers of 2 to 5 fish from each acclimated or acclimatized group were pooled to provide the homogenate for each group of assays. Due to the amount of homogenate available, the Km values for *Catostomus* and *Esox* were determined only once at each temperature. Most *Carassius* Km values were determined using two different homogenates (single assays of each) at each assay temperature, and for these determinations, the mean ± 1 SEM is shown.

	Carassius		*Catostomus*		*Esox*	
Assay T	Warm	Cold	Summer	Winter	Summer	Winter
22 C	102 ± 6	102 ± 8	124	125	60	61
12 C	62	54 ± 4	80	80	44	44
5 C	40	43 ± 2	72	75	40	41
2 C	39 ± 3	39 ± 2	--	49	--	42
0 C	--	--	--	82	--	58

Figure 1A (Upper). The Michaelis constant, Km(G6P), of goldfish (*Carassius*) liver G6PD plotted as a function of assay temperature. There were no differences in the Km values of enzymes from warm- and cold-acclimated fish (Table 1), hence the mean Km of all assays conducted at each temperature is shown. The number of determinations at each temperature is shown in Table 1.

Figure 1B (Lower). The Michaelis constant, Km(G6P), of white sucker (*Catostomus*) and northern pike (*Esox*) liver G6PD plotted as a function of assay temperature. There were no differences in the Km values of enzymes from winter and summer caught fish (Table 1), hence the mean Km of all assays conducted at each temperature is shown. The number of determinations at each temperature is shown in Table 1.


● Catostomus
▲ Esox
$K_{m\,(G6P)}$ $M \times 10^5$
0
2
4
6
8
10
12
14
0
5
10
15
20
25
TEMPERATURE, °C


Carassius
$K_{m\,(G6P)}$ $M \times 10^5$
0
2
4
6
8
10
12
0
5
10
15
20
25
TEMPERATURE, °C

increase in the apparent E-S affinity due to decreasing temperature has been termed positive thermal modulation by Somero (1969) and co-workers. The white sucker G6PD showed extensive positive thermal modulation between 22 C and 12 C (Km decreased from approx. 120 μM to 80 μM) and again below 5 C (Fig. 1B); negative thermal modulation, a decrease in apparent E-S affinity, occurred near 0 C. Pike Km(G6P) showed much less change with temperature than the Km's of the other species (Fig. 1B); below 1.8 C there was negative thermal modulation.

B. THE EFFECT OF TEMPERATURE ON NADP$^+$-DEPENDENT G6PD KINETICS

Michaelis constants for the apparent affinity of sucker and pike G6PD for NADP$^+$ (Table 2) were determined from double-reciprocal plots (Figs. 2A, B). No differences in the Km values were seen when the Km(NADP$^+$) was determined at 22 C and 2 C, nor were there major interspecific differences in the values (Table 2). Seasonal comparisons were not made. NADP$^+$ availability (Kather et al., 1972b), and the affinity of G6PD for this coenzyme (Greenbaum et al., 1971), are the rate-limiting factors for the G6PD reaction.

When NADP$^+$ was present at low concentrations (<15 μM), and the assay temperature was 2 C, there were upward curvatures in the double-reciprocal plots (Figs. 2A, B). These particular deviations from the expected linear relationship probably indicate interactions between binding sites (Bonsignore et al., 1970), and have been observed in kinetic studies of mullet fish G6PD (Hochachka and Hochachka, 1973).

Table 2. Kinetic constants for goldfish (*Carassius*), white sucker (*Catostomus*), and northern pike (*Esox*) liver G6PD at assay temperatures of 22 C (±1 C) and 2 C (±0.5 C). The Km(G6P) of each species is the mean of all assays conducted at either 22 C or 2 C (from Table 1). The Km($NADP^+$) and Ki(NADPH) for *Catostomus* and *Esox* were determined once at each temperature as described in Figures 2A, B and Appendix IV, Figures 1A, B. Goldfish Ki(NADPH) was determined (once) with a liver homogenate from warm-acclimated fish. All values are expressed as micromoles/liter.

	Kinetic constants for G6PD			
	Assay T (C)	Km(G6P)	Km($NADP^+$)	Ki(NADPH)
Carassius	22	102	--	25
Carassius	2	39	--	--
Catostomus	22	124	14	18
Catostomus	2	49	15	32
Esox	22	60	20	36
Esox	2	42	20	46

Figure 2A (Upper). Double-reciprocal plot showing the effect of different $NADP^+$ concentrations on the activity of white sucker (*Catostomus*) liver G6PD. G6P concentration was 0.2 mM. The homogenate was from May caught fish. The Km($NADP^+$) values are listed in Table 2. Regression equations are:

(22 C) 1/velocity = 0.5613 + 0.0079 (1/mM $NADP^+$)

(2 C) 1/velocity = 4.8935 + 0.0776 (1/mM $NADP^+$)

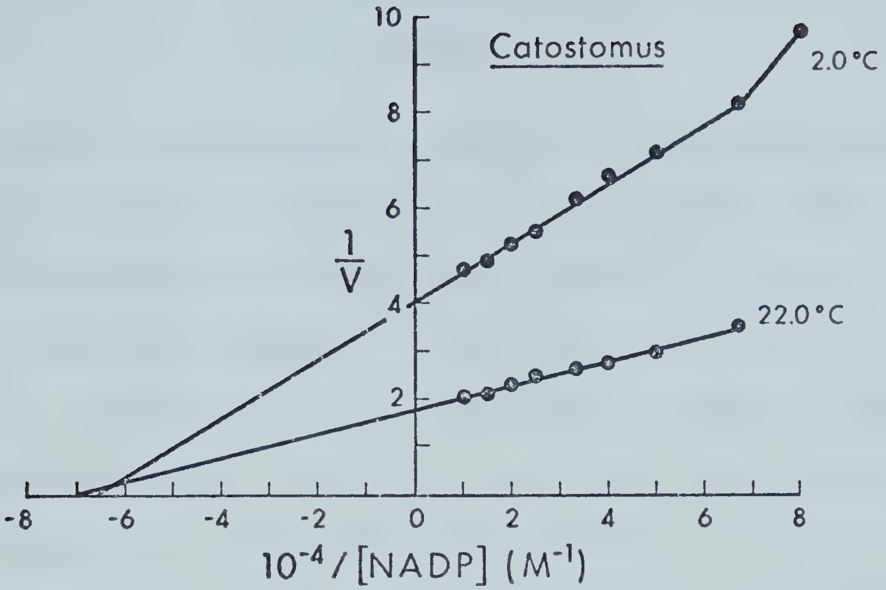
Velocity x 10^4 = nmol NADPH produced/min/gram wet weight of liver

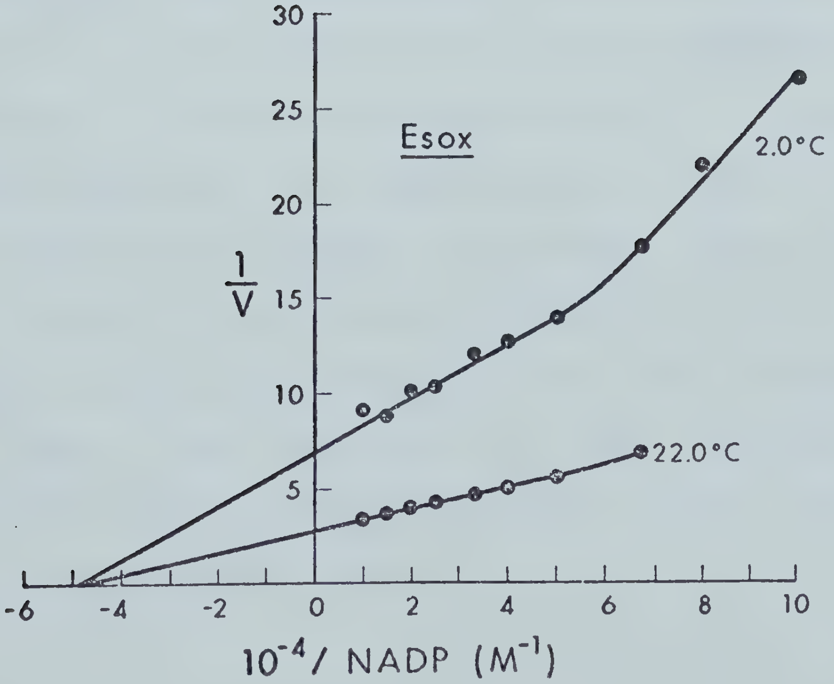
Figure 2B (Lower). Double-reciprocal plot showing the effect of different $NADP^+$ concentrations on the activity of northern pike (*Esox*) liver G6PD. G6P concentration was 0.2 mM. The homogenate was from July (summer) caught fish. The Km($NADP^+$) values are listed in Table 2. Regression equations are:

(22 C) 1/velocity = 14.3737 + 0.2872 (1/mM $NADP^+$)

(2 C) 1/velocity = 108.5147 + 2.2424 (1/mM $NADP^+$)

Velocity x 10^4 = nmol NADPH produced/min/gram wet weight of liver


Catostomus
10
8
6
4
2
1/V
2.0°C
22.0°C
-8
-6
-4
-2
0
2
4
6
8
10⁻⁴/[NADP] (M⁻¹)


Esox
30
25
20
15
10
5
1/V
2.0°C
22.0°C
-6
-4
-2
0
2
4
6
8
10
10⁻⁴/ NADP (M⁻¹)

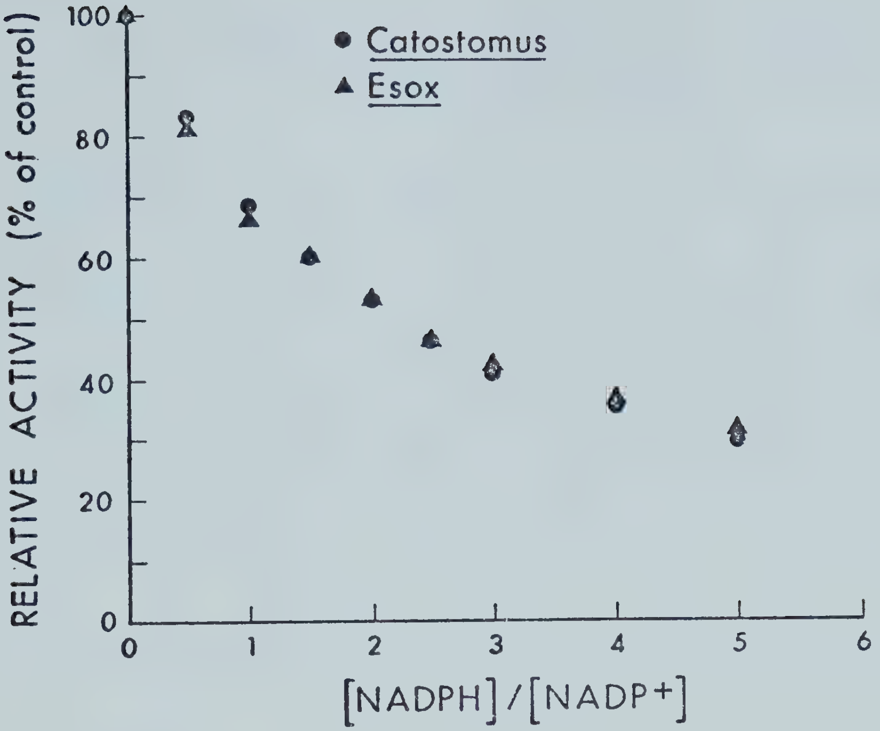
C. THE EFFECT OF TEMPERATURE ON INHIBITION OF G6PD KINETICS

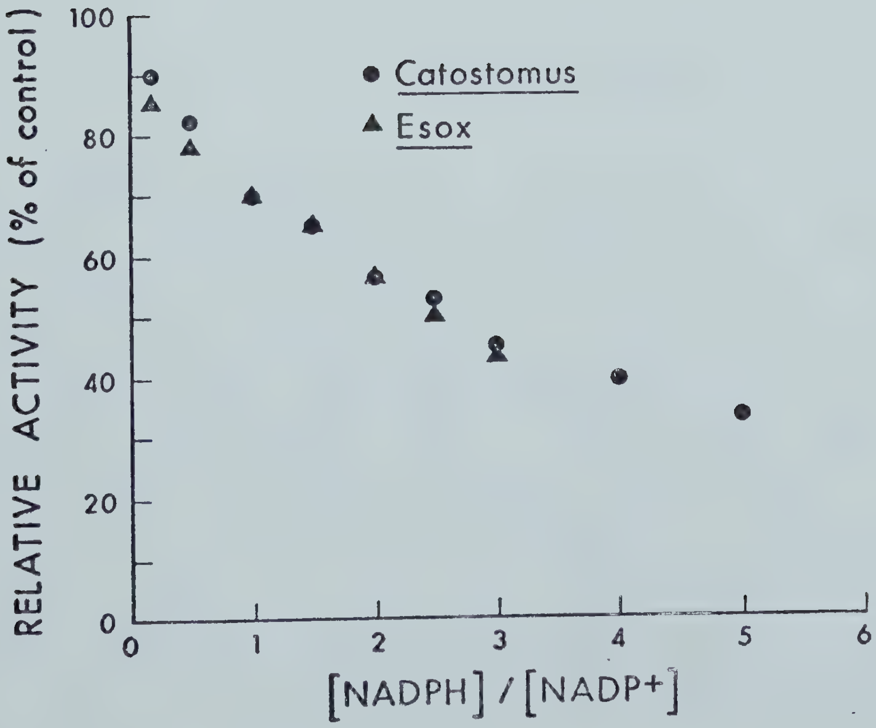
Of the possible physiological inhibitors examined (Appendix IV), the most potent inhibitor of G6PD was NADPH. The Ki(NADPH) values (Table 2) were determined from Dixon plots (Appendix IV) (Dixon, 1953). The Ki(NADPH) was measured against the $NADP^+$-dependent activity of sucker and pike G6PD. Inhibition of the goldfish enzyme by NADPH was measured against substrate-dependent activity. In both cases, the inhibition was assumed to be competitive (Passonneau et al., 1966). For sucker and pike G6PD, the Ki(NADPH) at 2 C was higher than the Ki at 22 C (Table 2), indicating, that at low concentrations (<50 μM) of NADPH, there was somewhat less inhibition at low temperatures.

The inhibition based upon $NADPH/NADP^+$ ratios is shown in Figures 3A, B. The G6PD reaction was 50% inhibited when the $NADPH/NADP^+$ ratio was approximately 2.5, and 70% inhibited at a ratio of 5. Extrapolation from the data in Figures 3A, B (by linear regression equations from the Dixon plots in Appendix IV) indicated that the sucker and pike G6PD reaction will be about 95% inhibited when $NADPH/NADP^+$ equals 50. Fifty is the approximate physiological $NADPH/NADP^+$ ratio under lipogenic conditions in the rat liver (Greenbaum et al., 1971). The amount of inhibition by NADPH, based upon $NADPH/NADP^+$ ratios, did not appear to change with temperature (Figs. 3A, B).

Figure 3A (Upper). Inhibition of white sucker (*Catostomus*) and northern pike (*Esox*) liver G6PD by varied NADPH/NADP$^+$ ratios. The G6P concentration was 0.2 mM; NADP$^+$ concentration was 0.05 mM; and NADPH concentrations were 0.025 to 0.25 mM. The homogenates used are the same as in Figures 2A and 2B. Assay temperature was 22.0 C.

Figure 3B (Lower). Inhibition of white sucker (*Catostomus*) and northern pike (*Esox*) liver G6PD by varied NADPH/NADP$^+$ ratios as described in Figure 3A. Assay temperature was 2.0 C.


RELATIVE ACTIVITY (% of control)
100
80
60
40
20
0
0
1
2
3
4
5
6
[NADPH]/[NADP+]
● Catostomus
▲ Esox


RELATIVE ACTIVITY (% of control)
100
80
60
40
20
0
0
1
2
3
4
5
6
[NADPH]/[NADP+]
● Catostomus
▲ Esox

D. THE EFFECT OF TEMPERATURE ON SUBSTRATE-DEPENDENT 6PGD KINETICS

Michaelis constants, Km(6PG) for 6PGD, were determined from double-reciprocal plots (Appendix III) and are shown as a function of temperature in Table 3 and Figure 4A for goldfish, and in Figure 4B for suckers and pike. There did not appear to be any acclimation-dependent differences in Km values for goldfish 6PGD (Table 3), suggesting that this enzyme, as well as G6PD, undergoes no qualitative changes in response to temperature acclimation. White sucker and northern pike 6PGD enzymes were examined from only one homogenate for each species, so seasonal comparisons could not be made.

The Km(6PG) values in all three species were nearly independent of assay temperature (Figs. 4A, B). The Km changed by no more than 10 μM over a 20 C temperature range in any of the species.

E. ACTIVATION ENERGIES

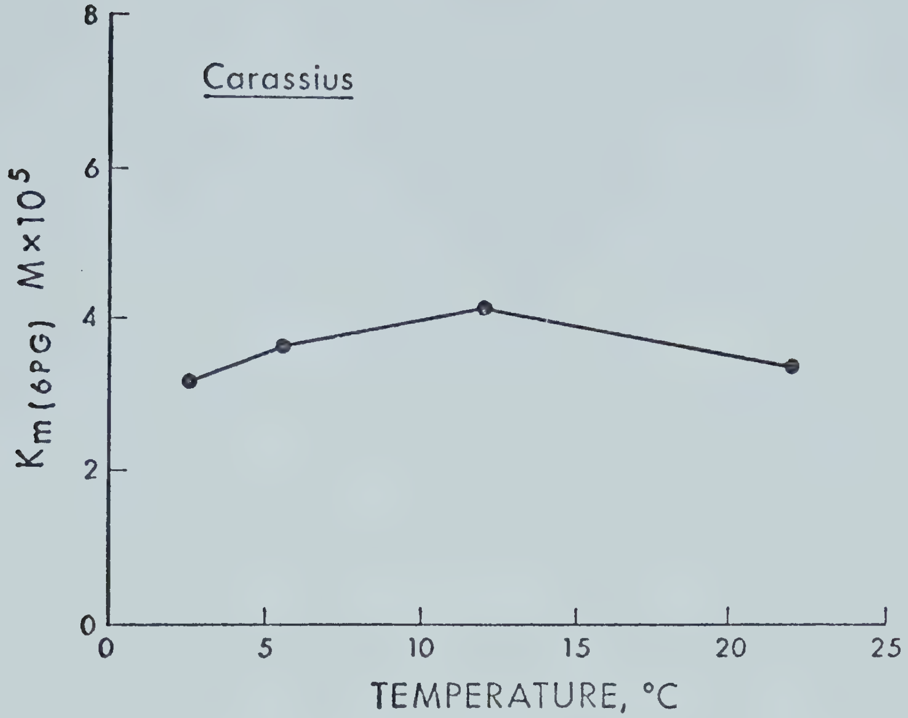
Arrhenius plots of Vmax vs. the reciprocal of the absolute temperature (apparent energy of activation, Ea) were non-linear for all G6PD enzymes examined (sucker and pike G6PD, Fig. 5A). Goldfish Ea values (Table 4) were calculated from Arrhenius plots. The transition temperature (Dixon and Webb, 1964, p. 158) for both sucker and goldfish G6PD was around 5 C, while that of pike G6PD was near 12 C. The apparent Ea values were similar for all three species, except that the goldfish G6PD showed a substantially lower Ea below 5 C compared to the other fish. No seasonal or acclimation-dependent changes in Ea were observed (Fig. 5A, Table 4).

Table 3. Michaelis constants, Km(6PG), for goldfish (*Carassius*) liver 6PGD from warm- (W, 20 C) and cold- (C, 2 C) acclimated groups of fish. Assay temperatures are within ±1.5 C. Km values were determined from double-reciprocal plots (Appendix III), and are expressed as micromoles of substrate per liter. The livers of 3 to 5 temperature-acclimated fish were used for each homogenate. At each temperature, the different Km values were determined using different homogenates.

Assay T	Km(6PG)	Mean Km ± SEM	T-acclimated
22 C	30	34 ± 3	W
	31		C
	32		C
	44		C
12 C	37	42 ± 3	W
	42		W
	54		C
	36		C
	41		C
5 C	42	38 ± 2	W
	38		C
	33		C
2 C	31	32 ± 1	W
	32		C

Figure 4A (Upper). The Michaelis constant, Km(6PG), of goldfish (*Carassius*) liver 6PGD plotted as a function of assay temperature. There were no differences in the Km values of enzymes from warm- and cold-acclimated fish (Table 3), hence the mean Km of all assays conducted at each temperature is shown.

Figure 4B (Lower). The Michaelis constant, Km(6PG), of white sucker (*Catostomus*) and northern pike (*Esox*) liver 6PGD plotted as a function of assay temperature. The sucker homogenate was from May caught fish, and the pike homogenate was from July caught fish. One determination was made at each temperature.


Carassius
$K_{m\,(6PG)}$ M×10^5
0
2
4
6
8
0
5
10
15
20
25
TEMPERATURE, °C

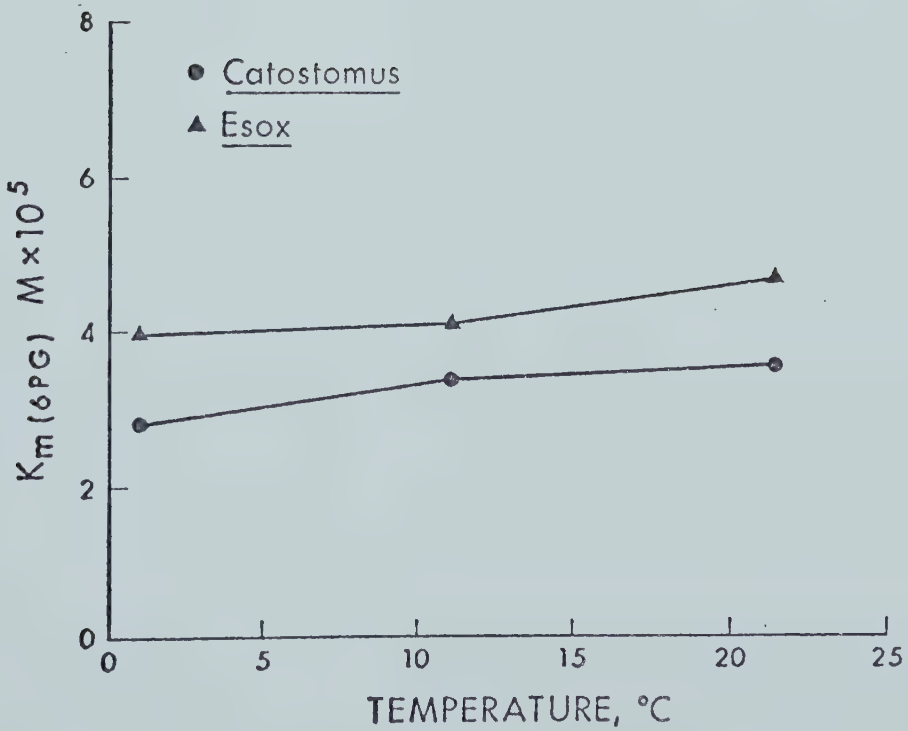
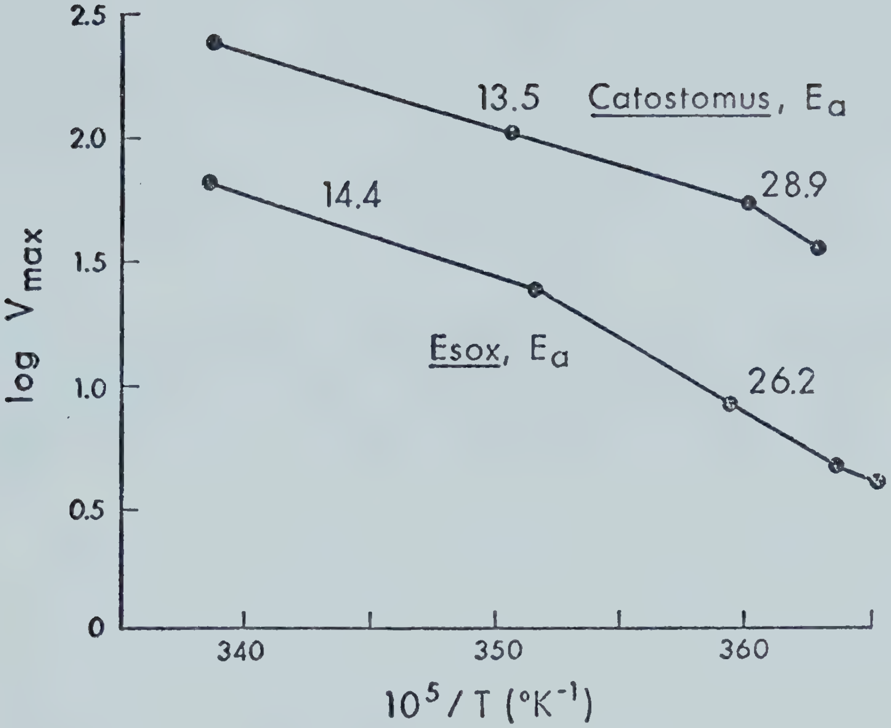

● Catostomus
▲ Esox
$K_{m\,(6PG)}$ M×10^5
0
2
4
6
8
0
5
10
15
20
25
TEMPERATURE, °C

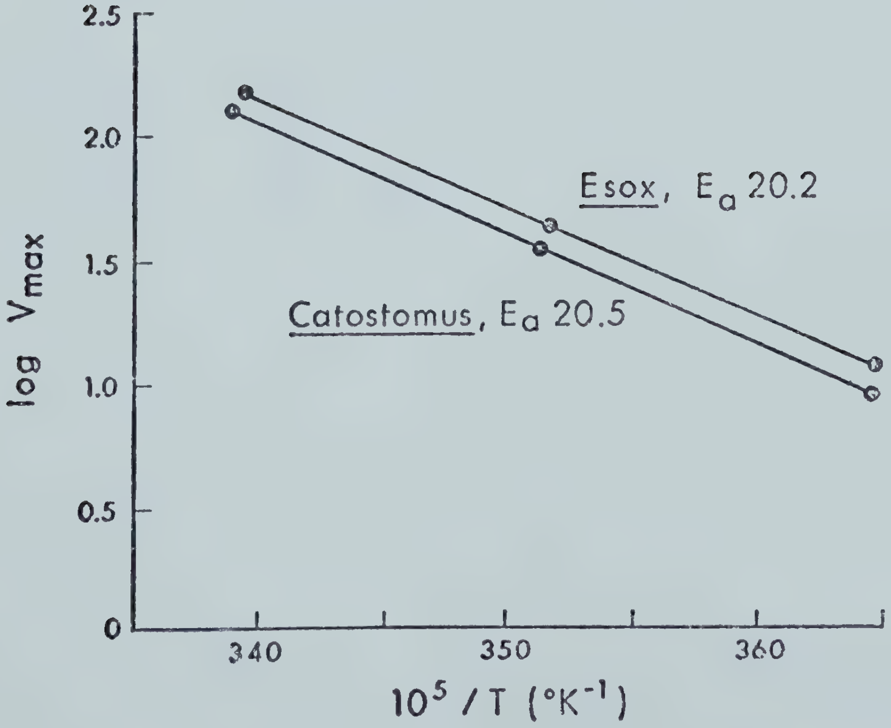
Figure 5A (Upper). Arrhenius plot of Vmax (Table 5) vs. the reciprocal of the absolute temperature of the enzyme assay for white sucker (*Catostomus*) and northern pike (*Esox*) liver G6PD. Energy of activation (Ea) values are expressed as Kcal/mole. Values from winter liver homogenates are plotted. Values from summer homogenates are listed below:

	T (C)	$10^5/T$ (K^{-1})	Ea (Kcal/mole)
Catostomus	23.0-13.0	337.6-349.5	14.3
	13.0- 5.7	349.5-358.6	14.4
Esox	22.0-13.0	338.8-349.5	15.9
	13.0- 5.5	349.5-358.9	27.1

Figure 5B (Lower). Arrhenius plot of Vmax (Table 6) vs. the reciprocal of the absolute temperature of the enzyme assay for white sucker (*Catostomus*) and northern pike (*Esox*) liver 6PGD. Energy of activation (Ea) values are expressed as Kcal/mole. The sources of the enzymes are listed in Table 6.



2.5
2.0
1.5
1.0
0.5
0
log V$_{max}$
13.5
Catostomus, Ea
28.9
14.4
Esox, Ea
26.2
340
350
360
10^5 / T (°K^{-1})



2.5
2.0
1.5
1.0
0.5
0
log V$_{max}$
Esox, Ea 20.2
Catostomus, Ea 20.5
340
350
360
10^5 / T (°K^{-1})

Table 4. Energy of activation (Ea) values for goldfish (*Carassius*) liver enzymes from warm- (W, 20 C) and cold- (C, 2 C) acclimated fish. The Ea values within each temperature range were ±15% of the mean value over the entire temperature range.

Enzyme	T range (C)	$10^5/T$ (K^{-1})	Ea (Kcal/mole)	T-acclimated
G6PD	22.0-5.0	338.8-359.5	12.6	W
"	22.0-4.8	338.8-359.8	11.7	C
"	22.0-5.0	338.8-359.5	15.1	C
G6PD	5.0-2.5	359.5-362.8	20.0	W
"	4.8-2.3	359.8-363.0	20.3	C
"	5.0-2.0	359.5-363.4	19.9	C
6PGD	23.5-5.2	337.1-359.3	12.1	W
"	22.0-6.0	338.8-358.2	12.8	C
6PGD	5.2-2.5	359.3-362.8	20.4	W
"	6.0-2.8	358.2-362.4	20.6	C

Both the sucker and pike 6PGD enzymes exhibited linear Arrhenius plots from 22 C to 1 C (Fig. 5B). The apparent Ea of goldfish 6PGD (Table 4) was identical to the energy of activation values of the other species (Fig. 5B) at temperatures below 5 C, but at higher temperatures it was much lower. No acclimation-dependent changes in Ea values of goldfish 6PGD were observed.

F. QUANTITATIVE ASPECTS OF G6PD AND 6PGD ACTIVITY

The most distinctive interspecific differences between the pentose phosphate pathway dehydrogenases were quantitative. The quantitative estimates of enzyme activity should be viewed with some caution, however, because of the possible loss of activity due to the homogenization procedure (p. 8,9) and the resultant presence of catheptic enzymes in the homogenate. The maximal catalytic potential (Vmax) of the G6PD and 6PGD enzymes for all three species is shown in Tables 5, 6. The G6PD activity of sucker and goldfish liver homogenates was, with one exception, at least ten times greater than the G6PD activity of pike homogenates, whether activity was expressed per gram wet weight of liver tissue or per milligram protein (Table 5, Fig. 6). The interspecific differences in 6PGD activity (Table 6) are similar but less dramatic. The highest activities of 6PGD were found in goldfish liver homogenates, and the lowest, in pike homogenates.

Differences in enzyme activities per milligram of protein were naturally dependent on the concentration of protein in the homogenates. In both sucker and pike, homogenates from the livers of winter-acclimatized fish had slightly less protein (Table 5). In goldfish, this was

Table 5. The maximum velocities (Vmax) of liver G6PD enzymes from goldfish (*Carassius*), white suckers (*Catostomus*), and northern pike (*Esox*). The Vmax was determined from double-reciprocal plots (Appendix III). At each temperature, the different Vmax values represent different homogenates. Velocity is expressed as nmol NADPH produced per minute.

Assay T (C)	Species	nmol/g liver	nmol/mg protein	µg protein/ml/1% homogenate
22.0	*Catostomus*[a]	14,522	405	356
23.0	*Catostomus*[b]	14,856	338	437
22.2	*Esox*[a]	1,025	49	210
22.0	*Esox*[b]	827	29	288
22.0	*Carassius*[c]	23,119	348	664
22.0	*Carassius*[c]	6,984	129	542
22.0	*Carassius*[d]	9,405	216	437
22.0	*Carassius*[d]	10,341	331	312
12.0	*Catostomus*[a]	6,655	187	356
13.0	*Catostomus*[b]	6,300	144	437
11.3	*Esox*[a]	405	19	210
13.0	*Esox*[b]	352	12	288
11.5	*Carassius*[c]	8,505	128	664
12.0	*Carassius*[c]	3,876	102	378
11.5	*Carassius*[d]	5,421	190	286
4.5	*Catostomus*[a]	3,396	99	344
5.7	*Catostomus*[b]	3,257	75	437
5.0	*Esox*[a]	142	7	210
5.5	*Esox*[b]	98	3	288
4.5	*Carassius*[c]	4,775	72	668
4.8	*Carassius*[c]	2,034	54	378
5.0	*Carassius*[d]	2,797	98	286
2.3	*Catostomus*[a]	2,207	64	344
1.8	*Esox*[a]	77	4	210
2.1	*Carassius*[c]	3,222	48	668
2.3	*Carassius*[c]	1,457	39	378
2.0	*Carassius*[d]	2,075	73	286
0.0	*Catostomus*[e]	2,030	---	---
0.6	*Esox*[a]	66	3	210

[a] Homogenate from March (winter) fish.

[b] Homogenate from July (summer) fish.

[c] Cold-acclimated fish.

[d] Warm-acclimated fish.

[e] Homogenate from May fish.

Table 6. The maximum velocities (Vmax) of liver 6PGD enzymes from goldfish (*Carassius*), white suckers (*Catostomus*), and northern pike (*Esox*). Other details as described in Table 5.

Assay T (C)	Species	nmol/g liver	nmol/mg protein	µg protein/ml/1% homogenate
21.7	*Catostomus*[a]	2,010	---	---
21.3	*Esox*[b]	952	---	---
22.0	*Carassius*[c]	5,017	124	404
22.0	*Carassius*[c]	2,978	68	479
23.5	*Carassius*[d]	3,950	93	427
21.8	*Carassius*[d]	4,070	112	364
11.4	*Catostomus*[a]	580	---	---
11.1	*Esox*[b]	289	---	---
12.0	*Carassius*[c]	2,572	64	404
11.8	*Carassius*[c]	1,172	25	479
12.5	*Carassius*[d]	1,867	44	427
11.8	*Carassius*[d]	1,695	47	364
6.0	*Carassius*[c]	1,432	35	404
5.2	*Carassius*[d]	1,027	24	427
5.1	*Carassius*[d]	981	27	364
1.1	*Catostomus*[a]	143	---	---
1.0	*Esox*[b]	75	---	---
2.8	*Carassius*[c]	926	23	404
2.5	*Carassius*[d]	716	17	427

[a] Homogenate from May fish.

[b] Homogenate from July (summer) fish.

[c] Cold-acclimated fish.

[d] Warm-acclimated fish.

Figure 6. The effect of temperature on the activity of goldfish (*Carassius*), white sucker (*Catostomus*), and northern pike (*Esox*) liver G6PD. Activity was calculated from the data presented in Figures 1-3, Appendix III. G6P concentration was 0.2 mM, $NADP^+$ was saturating. Activity of sucker and pike summer fish homogenates is shown at 12 C and above; activity of winter homogenates is shown below 12 C.

The effect of seasonal changes in liver weight on total NADPH production in the northern pike liver (axis on the right) is illustrated with the broken (- - -) line. The liver weights used are:

22, 12 C = 13 g

5 C = 15 g

1.8 C = 17-24 g (lower, upper broken lines, respectively)

0.6 C = 17-24 g (" " " " ")

(Weights are from Medford, 1976; and personal observations.)

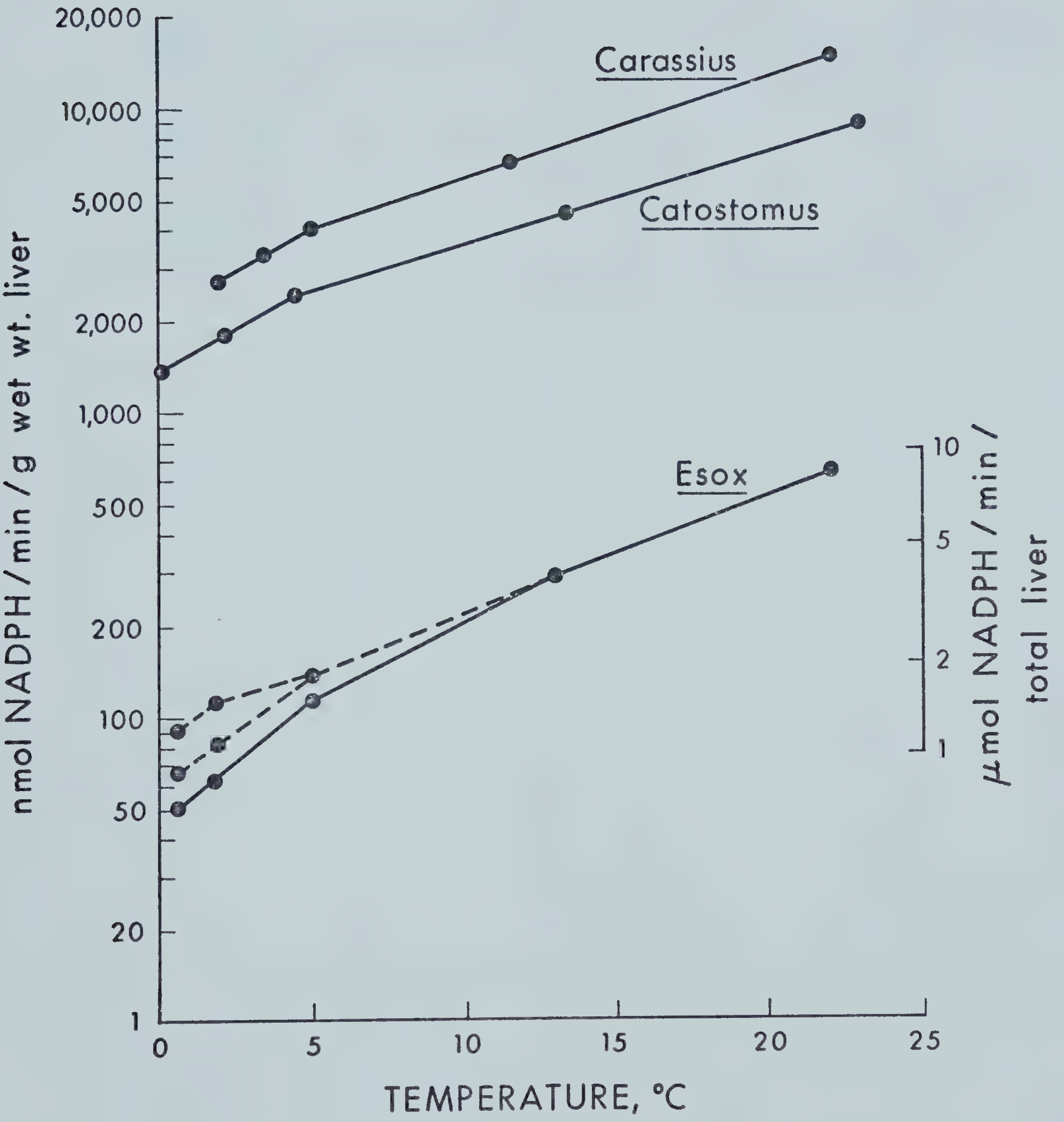

20,000
10,000
5,000
2,000
1,000
500
200
100
50
20
1
nmol NADPH / min / g wet wt. liver
Carassius
Catostomus
Esox
10
5
2
1
μmol NADPH / min / total liver
0
5
10
15
20
25
TEMPERATURE, °C

Table 7. Activity of some lipogenic or NADPH producing enzymes from white sucker (*Catostomus*) tissues. G6P and 6PG concentrations are 0.1 mM; all other concentrations and conditions are as described in the Materials and Methods section. The temperature of all assays was 22 C (±1.0 C). Activity is expressed as nmol NADPH produced/min/gram wet weight of tissue. Homogenates consisted of 2 to 5 pooled tissues. Assays were performed in duplicate and the mean values are presented. Individual assays were no more than ±5% from the mean value. Tr = trace, less than 15 nmol NADPH produced/min/gram.

	G6PD	6PGD	ME	ICD	αGPD
Liver[1]	8,296	1,592	139	3,473	483
Liver[2]	6,653	---	---	---	---
Liver[3]	6,462	---	---	---	---
White Muscle	Tr	19	58	2,662	636
Heart	0	199	54	18,183	31
Visceral Fat	820	193	0	567	97
Gill	617	212	0	386	20
Brain	424	300	0	2,508	241

[1] Fish caught - May 1975.

[2] Fish caught - July 1974.

[3] Fish caught - March 1974.

reversed, with liver homogenates from cold-acclimated fish containing somewhat greater protein concentrations (Tables 5, 6).

There appeared to be no quantitative differences in sucker G6PD activity per gram liver (Table 7) when March and July fish homogenates were compared. However, there was increased G6PD activity in the May caught fish which were migrating upstream to spawn. Goldfish G6PD activity was somewhat variable (Table 8). Cold-acclimated goldfish were kept under shorter daylight photoperiods than warm-acclimated goldfish (p. 6). This has been shown to elicit the greatest differences in G6PD between cold- and warm-acclimated mullet fish, *Mugil cephalus* (Hochachka and Hochachka, 1973). However, goldfish G6PD activity showed no consistent acclimation-dependent differences (Table 8). Differences in G6PD activity among different shipments of fish were evident (Table 8). Goldfish 6PGD activity was less variable than G6PD activity, but showed no acclimation-dependent differences either (Table 8). Cold-acclimated goldfish that were fasted for one month or seven months showed no detectable decrease in G6PD or 6PGD activity (Table 8), suggesting that the variable activity of these enzymes in goldfish liver (Table 8) was probably not due to differences in food intake. Pike G6PD activity per gram liver was about 20% greater in winter than in summer caught animals (Table 9). This may have been only a transient increase due to feeding, but because the homogenates were made from pooled livers, the activities represent the average of several individuals, which suggests the measured seasonal differences in enzyme activity were a real seasonal phenomenon. Due to the number of fish available, no preliminary tests were conducted to determine the variability between pooled liver

Table 8. Activity of some lipogenic or NADPH producing enzymes from goldfish (*Carassius*) tissues. Other details as in Table 7. Activity is expressed as nmol NADPH produced/min/gram wet weight of tissue.

	G6PD	6PGD	ME	ICD	αGPD	Year of shipment
Liver[1]	9,164	2,700	350	2,195	6,096	1974
Liver[1]	---	3,837	---	---	4,726	1974
Liver[2]	9,900	4,196	488	3,730	5,367	1974
Liver[3]	18,681	4,703	1,130	4,437	8,655	1975
Liver[4]	---	---	---	---	4,438	1974
Liver[4]	5,182	3,128	---	---	---	1973
Liver[4]	5,175	3,003	---	---	---	1973
Liver[5]	10,994	---	---	---	---	1974
Liver[5]	4,942	3,816	---	---	---	1973
Liver[5]	3,858	2,070	---	---	---	1973
White Muscle	Tr	29	63	1,119	1,460	1974
Visceral Fat[2]	45	15	0	0	54	1974
Visceral Fat[3]	0	0	0	0	---	1975

[1] Cold-acclimated fish, fasted one month.

[2] Cold-acclimated fish, fasted seven months.

[3] Warm-acclimated fish, fed in excess.

[4] Warm-acclimated fish, fed.

[5] Cold-acclimated fish, fed.

Table 9. Activity of some lipogenic or NADPH producing enzymes from northern pike (*Esox*) tissues. Other details as in Table 7. Activity is expressed as nmol NADPH produced/min/gram wet weight of tissue.

	G6PD	6PGD	ME	ICD	αGPD
Liver[1]	530	1,050	0	4,630	322
Liver[2]	515	--	--	--	--
Liver[3]	644	--	--	--	--
White Muscle	Tr	40	39	707	2,369

[1] Fish caught - July 1974; enzymes assayed June 1975.

[2] Fish caught - July 1974.

[3] Fish caught - March 1974.

homogenates representing different groups of naturally acclimatized animals. However, the two pike liver homogenates from animals collected in July, 1974 (Table 9), do represent different groups of fish (3-4 individuals), with the tissues of one group frozen for nearly a year before the homogenate was made. The virtually identical G6PD activity of the two homogenates suggests that only a minor amount of variability may exist between homogenates from different animals collected at the same time of year.

The overall effect of temperature on liver G6PD activity from the three species is shown in Figure 6. The greater the slope, the more sensitive the activity was to changes in temperature. Figure 6 also shows how seasonal changes in the weight of the pike liver (Medford, 1976) affect total liver NADPH production.

G. TISSUE PROFILES OF ENZYME ACTIVITY

The activities of five lipogenic or NADPH generating enzymes from a variety of tissues are presented in Tables 7-9. Some of the more important findings are:

1. The highest activities of the NADPH generating enzymes (excluding $NADP^+$-isocitrate dehydrogenase in heart muscle, and pike malic enzyme) were found in the liver of all three species.

2. No malic enzyme activity was detectable in pike liver, suggesting an absence of the complete malate cycle (Flatt, 1970) in this tissue. In the livers of the other species studied, malic enzyme was present, but exhibited no more than 6% of the activity of G6PD, or 24% of the activity

of 6PGD.

3. NADP$^+$-isocitrate dehydrogenase showed moderate to high activity in nearly all tissues, including those, such as white muscle and heart, in which the activity of other NADPH producing enzymes was low.

4. Tissue patterns of *alpha*-glycerophosphate dehydrogenase activity showed the most variability among the three species. Goldfish exhibited high activity in liver and muscle, while pike had relatively low activity in liver, but high activity in muscle. Suckers showed comparatively low to moderate activity in liver and muscle, and had some activity in visceral fat preparations.

5. Visceral fat from white suckers exhibited low NADPH generating activity compared to the liver. Virtually no NADPH generating activity was detectable in goldfish visceral fat. The visceral fat examined was the loose fat associated with the liver or lying along the intestine, or, in suckers, fat located just inside the ventral abdominal wall. Variable, usually small (<0.2% somatic body weight, Medford, 1976) amounts of fat are sometimes found along the intestine in pike, but these depots were not examined for enzyme activity.

DISCUSSION

I. THE EFFECT OF TEMPERATURE ON G6PD AND 6PGD

A. The Effect of Temperature on Substrate-Dependent G6PD Kinetics

i. The absence of qualitative changes. There were no acclimation-dependent or seasonal differences in the Km-temperature relationships of the G6PD (and 6PGD, Table 3) enzymes studied (Table 1). The absence of differences in the Km values strongly suggests that there are no seasonal or temperature-dependent isozymic changes. This was expected, since a majority of teleost species, including goldfish (Wilson et al., 1973), do not normally exhibit isozymic changes in response to acclimation and acclimatization (Somero, 1975a). This absence of qualitative change has been termed the "eurytolerant protein" strategy (Somero, 1975a), and implies that most enzymes, such as G6PD from a number of teleost species (Mester et al., 1972; Somero, 1975a; Table 1, present work), have properties which allow for proper function at all temperatures the organism normally encounters.

However, isozymic changes in the enzyme complement of a tissue due to acclimation or acclimatization have been demonstrated in a number of fish (see reviews by Hochachka and Somero, 1973; Hazel and Prosser, 1974). These isozymic changes are generally restricted to salmonids (Somero, 1975a), although the acclimation-dependent changes in G6PD isozymes exhibited by mullet fish (Hochachka and Hochachka, 1973) provide a relevant and somewhat unique example in a non-salmonid species.

ii. Temperature modulation. Since qualitative changes, based upon Km(G6P) values, did not occur seasonally in the G6PD enzymes examined (Table 1), one may ask whether the immediate or inherent effects of temperature on the enzyme affinity for substrate G6P (this section), coenzyme, $NADP^+$ (section B), or inhibitor, NADPH (section C), may be of physiological or rate-compensatory significance, and thus enable the enzymes to function effectively throughout the annual temperature cycle. Such changes have been observed in a number of poikilotherm enzymes (Somero, 1969).

The effect of temperature on the Km(G6P) was different for each species studied in the present work (Figs. 1A, B). In common with other poikilotherms studied (Somero, 1969; Robert and Gray, 1972b; Hochachka and Hochachka, 1973), the goldfish and suckers utilize temperature as a positive modulator of apparent enzyme-substrate affinity, over most of their temperature range, while the pike do not to any great extent. All three of the G6PD enzymes studied showed a minimum Km of 38-50 μM, which occurred at the lower end of the habitat temperature range (Figs. 1A, B). G6PD enzymes from other poikilotherms show similar Km values (45 μM, barracuda, *Sphyraena pinguis*, Shimeno and Takeda, 1972; 25 μM, king crab, *Paralithodes camtschatica*, Somero, 1969; 70 μM, mullet fish, *Mugil cephalus*, Hochachka and Hochachka, 1973; 41-50 μM, carp, grass carp, and eel, Nagayama et al., 1975b) at the lower end of their respective habitat temperature ranges. This suggests that proportional use of this enzyme (and of the pentose phosphate pathway) may be maximal at lower temperatures in poikilotherms. The assumption behind such a suggestion is that the Km occurs at, or slightly above, the substrate concentration in the cell, allowing for control by substrate availability, and

conversely, that *in vivo* concentrations are low and approximately equal the Km (Hochachka and Somero, 1973; Somero, 1975b). Comparison of G6PD apparent enzyme-substrate affinities with G6P concentrations reported in fish, mammals, and insects (a minimum of about 200 μM G6P, Appendix V), suggests that, for this enzyme, the assumption is not valid. It appears that the lowest Km(G6P) found in the present study (40-50 μM) is at least four to ten times lower than the G6P concentration in the cell (Appendix V). Therefore, the changes in Km(G6P) with temperature are probably not physiologically relevant in terms of metabolic control or rate-compensation. Previous temperature-dependent kinetic work on G6PD (Somero, 1969; Robert and Gray, 1972b; Hochachka and Hochachka, 1973) should be interpreted with due regard for this consideration.

The possibility of significant steady-state changes in G6P concentration with changes in temperature should not be overlooked, since an increase in substrate concentration could result in an increase in velocity as long as the enzyme was not completely saturated. If the sucker liver G6P concentration was 400 μM rather than 200 μM at 2.3 C, the velocity would increase by about 10%, as calculated from the equations in Appendix III. Freed (1971) found a lower G6P concentration (approx. 200 nmoles/gram) in the muscle of 15 C acclimated goldfish compared to 25 C acclimated fish (800 nmoles/gram). However, in the muscle of the goldfish orfe, *Idus idus*, acclimated to 6 C, the G6P concentration was more than double that found at moderate (10 C) or high (30 C) acclimation temperatures (Gronow, unpubl., the data are presented as a percentage of the control in Precht et al., 1973). Unfortunately, neither study measured G6P concentrations in the liver. Furthermore, it is not clear

how one would interpret data on G6P concentrations, if available, since, in the rat liver, G6P decreases in both starvation and a highly lipogenic nutritional state, when compared to the control value (Greenbaum et al., 1971).

B. The Effect of Temperature on NADP$^+$-Dependent G6PD Kinetics

It may be suggested that if modulation were of primary importance as a mechanism of temperature compensation in G6PD, then selective pressure (Crowley, 1975) would most likely result in adaptive changes in the affinity of the enzyme for its rate-limiting factor. Since NADP$^+$ availability has been shown to control the reaction velocity of G6PD in rat liver and adipose tissue (Greenbaum et al., 1971; Kather et al., 1972b), one might thus expect changes in the Km(NADP$^+$) with temperature. Figures 2A, B and Table 2 show Km(NADP$^+$) values of 14-20 μM in the fish presently under consideration. These values are virtually identical to those found in other teleosts (15-25 μM; barracuda, *Sphyraena pinguis*, Shimeno and Takeda, 1972; mullet fish, *Mugil cephalus*, Hochachka and Hochachka, 1973; and carp, grass carp, rainbow trout, eel, and yellowtail, Nagayama et al., 1975b) but somewhat higher than values reported for rat liver G6PD (6 μM, Sapag-Hagar et al., 1973). These Km values are in the middle of the *in vivo* NADP$^+$ concentration range (for laboratory rats, Bucher et al., 1964), and so small physiological increases in NADP$^+$ concentration, via its regeneration by reductive biosynthesis, would result in significant increases in reaction velocity. Thus, based upon the Km values, NADP$^+$ availability appears to be the controlling factor in the reaction rate of sucker, pike, and presumably goldfish G6PD.

However, one does not see changes in the Km($NADP^+$) due to temperature in either sucker or pike G6PD when assayed at 22 C and 2 C (Figs. 2A, B; Table 2). Hochachka and Hochachka (1973) also found very little thermal modulation (25-20 μM) of this parameter in mullet fish G6PD when measured at temperatures which were low to moderate (16-25 C) for this species. The possibility of seasonal changes in Km($NADP^+$) values was not examined because sufficient tissue samples were not available. However, it is unlikely that such changes occur because of the absence of seasonal changes in the Km(G6P) and because of the identical Km($NADP^+$) values at 22 C and 2 C when May (sucker) and July (pike) homogenates were assayed. Possible seasonal changes in $NADP^+$ concentrations have not been examined, but would probably be highly dependent upon the rates of reductive biosynthesis.

C. The Effect of Temperature on Inhibition of G6PD Kinetics

NADPH was the most potent physiological inhibitor of G6PD of those inhibitors tested in this study (Table 2; Appendix IV), and has been shown to be the primary physiological inhibitor of rat liver G6PD (Sapag-Hagar et al., 1973). The Ki for NADPH changed slightly (Table 2) with temperature in sucker and pike liver G6PD in such a way as to produce less inhibition at lower temperatures. However, if the NADPH concentration is calculated indirectly from known $NADP^+$ concentrations (Bucher et al., 1964; Greenbaum et al., 1971) and NADPH/$NADP^+$ ratios (>45, Greenbaum et al., 1971; >70, Krebs and Veech, 1969), then the NADPH concentration is ten or more times greater than the Ki(NADPH). Directly determined NADPH concentrations in laboratory rats are also at least ten times greater than the Ki(NADPH) (250-450 nmole/gram wet weight, Bucher

et al., 1964). Therefore, the small change in Ki(NADPH) (Table 2) would not greatly reduce the inhibition at low temperatures (compare Figs. 3A and 3B). From extrapolation of the regression equations from the Dixon plots (Appendix IV) one can show that, at a NADPH/NADP$^+$ ratio of 50, the G6PD reaction will be approximately 96% inhibited at 22 C, and approximately 95% inhibited at 2 C in suckers and pike. Therefore, only a minor rate-compensating effect can be attributed to temperature modulation of the inhibitor constant for NADPH.

In summary, the decrease in the G6PD reaction velocity due to temperature is not compensated for, except in a minor way, by a positive thermal modulation strategy in goldfish, suckers and pike, because, at least in suckers and pike, there is no adaptive change in the apparent affinity of G6PD for NADP$^+$, the physiological rate-limiting factor. Other immediate temperature-dependent changes in the affinity parameters, Km(G6P) and Ki(NADPH), are of only minor importance because the concentrations of G6P and NADPH in the cell are probably much higher than the Km or Ki.

It appears that if a substrate or coenzyme is rate-limiting for a particular reaction, then its apparent affinity for the enzyme is likely to be relatively independent of temperature over much of the enzyme's physiological temperature range (Moon, 1972; Somero, 1975b; Table 2, this work). Therefore, temperature compensation, as illustrated in the above discussion, is not "often effected" by changes in Km values (modulation strategy), since this would result in changes in regulatory function at different temperatures (Somero, 1975b). Regulatory function, the affinities of enzymes for their substrates and various modifiers, appears to be a conservative enzyme property, as illustrated by the

normally submaximal function of enzymes and the similarity in substrate and coenzyme affinities for homologous enzymes from a number of organisms living in different thermal environments (Somero, 1975b).

D. Energies of Activation, Ea, of G6PD

Temperature-dependent conformational changes which result in modulation of apparent enzyme-effector affinities were of only secondary importance for rate compensation (see above). Perhaps, then, the G6PD enzymes studied have an inherently high catalytic efficiency, Vmax (low energies of activation), or conformational changes take place which result in greater catalytic efficiency (lower Ea) at lower temperatures. Vroman and Brown (1963) have suggested that enzymes from cold-adapted organisms may have lower energies of activation than those from warm-adapted species, which would permit the maintenance of catalysis at low temperatures. Some enzymes from cold-adapted and antarctic fish do show reduced Ea values compared to the homologous enzymes from warm-adapted species (Somero, 1975b), but other enzymes do not (Hochachka and Somero, 1973). The energies of activation found in the present study (Fig. 5A, Table 4), though not especially low, were comparable to values of other poikilotherm G6PD enzymes (Robert and Gray, 1972b; Audilet and Gray, 1973b; Hochachka and Hochachka, 1973). The Arrhenius plots (Fig. 5A) were non-linear, and similar to those found with blue crab G6PD (Robert and Gray, 1972b). According to Baldwin and Hochachka (1970), non-linear Arrhenius plots are not unusual when both Vmax and Km change with temperature. The downward break or transition in the Arrhenius plots (Fig. 5A) suggests that temperature-dependent conformational changes affecting the catalytic efficiency (Vmax) take place at lower

temperatures, and that these changes result in the production of a poorer catalyst (higher Ea) rather than a better one.

E. Temperature-Dependent Kinetics of 6PGD; Control of the Pentose Phosphate Pathway

In the present study of 6PGD no temperature acclimation-dependent differences in apparent E-S affinity were observed (goldfish - Table 3), suggesting that, like G6PD, no isozymic changes occurred. There was a nearly complete absence of temperature-modulated changes in the apparent enzyme-substrate affinity (Figs. 4A, B). Based on previous arguments, the temperature insensitivity of the E-S affinity, and the reported *in vivo* 6PG concentrations (Appendix V) suggest substrate-dependent control of 6PGD, which is expected if flow through the PP pathway is controlled by $NADP^+$ availability at the preceding G6PD step. In addition, over the temperature range measured, the Ea values for 6PGD in the sucker and pike were relatively constant (Fig. 5B). It is not known why goldfish 6PGD should exhibit changes in Ea (Table 4), when its Km(6PG) shows temperature independence similar to the sucker and pike 6PGD enzymes.

So far, it has been assumed that the enzyme controlling the rate of metabolite flow through the oxidative portion of the pentose phosphate pathway is G6PD. This is an obvious assumption when considering such tissues as pike liver, where 6PGD activity greatly exceeds G6PD activity (Table 9), but it is not so obvious in sucker and goldfish liver, where G6PD activity greatly exceeds that of 6PGD (Tables 7, 8). In rat liver, the two major lines of evidence (from Greenbaum et al., 1971) pointing to control at the G6PD site are: that G6PD is a non-equilibrium reaction, while 6PGD is an equilibrium reaction; and that maximum

phenazine methosulfate stimulated flow through the pathway is directly related to the activity of G6PD, measured *in vitro*. However, Sapag-Hagar et al. (1973) have questioned, on the basis of an apparent imbalance of activities between G6PD and 6PGD, whether there is control at the G6PD site, and whether the oxidative PP pathway is an unbranched sequence.

A thorough study of the physiological control or inhibition of the 6PGD reaction was not undertaken in this work. It has been shown, in four of the five teleosts studied by Nagayama and Ohshimi (1975) and Nagayama et al. (1975b), that 6PGD has a greater affinity than G6PD for $NADP^+$, the rate-limiting coenzyme (Greenbaum et al., 1971). Therefore, G6PD will, in this work, continue to be considered the controlling enzyme of the PP pathway, although the low 6PGD activities (compared to G6PD) of sucker and goldfish liver do suggest consideration of an alternate interpretation.

F. Quantitative Changes in Enzyme Activity

There are two other mechanisms which goldfish, suckers, and pike can use to maintain or proportionally increase the rate of NADPH production via the PP pathway as the kinetic energy of the environment decreases. The enzyme activity or concentration can increase per gram of tissue, or the total enzyme activity of an organ can increase due to an increase in organ size. Quantitative changes in enzyme activity due to temperature acclimation or seasonal acclimatization have been shown for a number of enzymes and species, but, as Hochachka and Somero (1973) correctly observe, it is not known in most cases whether new enzyme forms are present or whether the enzyme concentration in the cell has actually increased. Table 9 shows that pike liver G6PD activity is about 20%

higher in winter than in summer caught fish. The pooled samples, the identical source of enzyme (male pike liver), and identical kinetic constants and Ea's (Table 1, Fig. 5A), suggest that there was, in fact, an actual increase in G6PD concentration in winter pike liver. Sucker liver G6PD (Table 7) and goldfish liver G6PD and 6PGD activity (Table 8) showed no quantitative change when winter and summer or cold- and warm-acclimated activities were compared, although goldfish homogenates showed considerable variability.

No consistent response has been found by other workers studying the dependence of PP pathway dehydrogenase activity on temperature acclimation. Ekberg (1962) found an increase in gill 6PGD activity and no change in gill G6PD activity when cold-acclimated crucian carp were compared to warm-acclimated ones. Lehmann (1970) found slight (16-17%) increases in G6PD and 6PGD activity in muscle in goldfish acclimated to 5 C compared to the 15 C acclimated group. Similar findings were reported by Kunnemann et al. (1971) for muscle G6PD from *Idus idus*. In the two latter papers, the variability in enzyme activity between individuals was high. Since the utilization of the PP pathway in muscle is extremely low (Green and Landau, 1965), it is uncertain whether the slight increases in muscle G6PD have any significance.

Braun et al. (1970) found increases in liver, muscle, and gill G6PD activity and decreases in intestinal G6PD activity in cold-acclimated bitterling, *Rhodeus amarus*. To this author's knowledge, theirs is the only reported increase in G6PD activity in fish liver due to cold-acclimation or acclimatization, except for the increase in northern pike G6PD activity in the present work (Table 9). It should be noted that malic enzyme, another enzyme producing reducing equivalents

for lipogenesis, showed substantially decreased activity in liver from cold-acclimated *Rhodeus*. Different enzymic responses to cold-acclimation in different species and organs (see Table 5 in Braun et al., 1970), differences in diet, and the often great physiological variability between different populations of fish, especially goldfish and carp (Wilson et al., 1973; see shipment date, Table 8), may be responsible for some of the differences observed among the studies cited above.

In contrast to either transient or seasonal increases in enzyme activity or concentration, another quantitative possibility is an increase in the size of the tissue concerned with the particular biochemical function. This strategy has been rather neglected by comparative biochemists, but can be very important from the animal's point of view. If the enzyme activity per gram stays constant, any increase in organ size, regardless of its etiology, will increase the total enzyme activity. Data from Medford (1976) show that (in Lac Ste Anne) the liver in winter caught pike is about twice as large as the liver in summer caught pike (see Fig. legend 6). Other species show seasonal changes in liver size as well (Inui and Ohshima, 1966). The increase or maintenance of enzyme activity per gram in the pike's enlarged winter liver suggests that, despite relatively low G6PD activity (Table 9), the PP pathway may play an important role in pike metabolism, and that this role may be especially important in winter, or is closely tied to liver function.

The potential significance on increases in pike liver size in temperature compensation by G6PD is illustrated in Figure 6. At 2 C (December data, Medford, 1976) a 17 g liver weight can be used for total

enzyme activity estimates, while at 0.6 C, a 24 g liver weight can be used for activity estimates (personal observations; early March data, Medford, 1976). Obviously, this is a simplification, since the liver is more or less continuously growing, and the temperature fluctuates somewhat. Nonetheless, using the simplifications, the increase in liver size over the winter might allow the rate of NADPH production by liver G6PD to be almost temperature-independent at low temperatures.

G. Summary and Conclusions

In summary, there are some species differences in the temperature-dependent properties of glucose-6-phosphate dehydrogenase. Temperature modulation of G6PD activity plays a tangible, but minor, role in rate compensation in suckers and goldfish. Quantitative seasonal changes in G6PD activity were found in northern pike, and appeared to be due primarily to changes in liver size.

This study has found: (1) no kinetic evidence (Table 1) of different isozymic forms of G6PD in warm- and cold-acclimated goldfish, or seasonally acclimatized white suckers and northern pike; and (2) rate-temperature plots (Fig. 6) of enzyme activity which show that none of the enzymic mechanisms present appear to provide a significant amount of rate compensation compared to the overall effect of temperature on the reaction rates. Thus, for these fish, the catalytic properties of the pentose phosphate pathway dehydrogenases do not appear to provide a mechanistic basis for increased PP pathway participation in carbohydrate catabolism. This is in agreement with the findings of Robert and Gray (1972a, b) on blue crab G6PD, but not with the view of Hochachka and Hochachka (1973) that temperature-dependent properties of mullet fish

G6PD do provide such a mechanism.

However, increased *de novo* lipogenesis from acetate does occur in cold-acclimated brook trout (Hochachka and Hayes, 1962), goldfish (Knipprath and Mead, 1968), and rainbow trout (Dean, 1969); although in cold-acclimated goldfish, the increased lipogenic rate is not reflected in the total lipid content (Knipprath and Mead, 1968; Appendix VI, present work). In view of the relationship between the NADPH generation and rates of lipogenesis, some proportionally increased pentose phosphate pathway utilization probably does occur in some winter acclimatized or cold-acclimated fish (certainly, in brook trout, Hochachka and Hayes, 1962), in spite of the absence of any temperature-dependent properties of the G6PD reaction which would provide a basis for this. Proportionally increased carbon flow through the PP pathway in most cold-acclimated fish probably occurs because of increased rates of lipogenesis which are due to the favorable energetic conditions in the cell at low temperatures. The probable increase in lipogenesis results in a decrease of the NADPH/$NADP^+$ ratio (Greenbaum et al., 1971) and the greater availability of $NADP^+$ for the G6PD reaction.

For goldfish that are cold-acclimated, the possible increase in 6PGD activity in the gill (Ekberg, 1962), would not result in increased flow through the PP pathway unless it was accompanied by increased G6PD activity due to the above-mentioned adjustments. It is possible that goldfish, at moderately low temperatures (around 5 C, as in most experiments) use a quantitative strategy. Due to the variability in the experimental animals and tissue preparations (Wilson et al., 1973; Table 8, present work), this mechanism of temperature compensation would be the

most difficult to consistently demonstrate experimentally. The qualitative strategy, if used, would not be as easily obscured by variability. Thus, this work shows that qualitative changes do not occur in goldfish PP pathway dehydrogenases, confirming the work of Wilson et al. (1973) on other goldfish enzymes.

II. THE PRODUCTION OF REDUCING EQUIVALENTS AND LIPOGENESIS

A. Sites of Lipogenesis Based on Tissue Enzyme Profiles

The activity of the pentose phosphate pathway dehydrogenases and malic enzyme can be positively correlated with the rate of *de novo* lipogenesis in rat liver (Pande et al., 1964; Leveille, 1970). Therefore, unless the sources of reducing equivalents for lipogenesis in fish tissues are quite different from those in mammalian tissues, it appears from the data in Tables 7-9 that the liver is probably the predominant organ of *de novo* lipogenesis in suckers, goldfish and possibly pike. The same enzyme pattern is also seen in bitterling, *Rhodeus amarus* (Braun et al., 1970). This is similar to findings in some birds (Goodridge and Ball, 1967; O'Hea and Leveille, 1969a) and in man (Shrago et al., 1971). In some other animals, such as the pig (O'Hea and Leveille, 1969b), and some ruminants (Ingle et al., 1972), adipose tissue is the primary organ of *de novo* lipogenesis. In frogs, both adipose tissue (corpora adiposa or fat body) and liver are important lipogenic organs (Baranska and Wlodawer, 1969).

The high lipogenic enzyme activity of the liver of suckers and goldfish (Tables 7, 8), coupled with the high lipid content of the liver relative to other tissues (Appendix VI), and the small quantity or absence of lipogenic "adipose tissue" (see below), indicates that, in these two teleost species, the liver probably functions as adipose tissue in the mammalian context. That is, the liver consists, in part at least, of tissue whose primary function is the storage of large quantities of lipid, much of which is enzymatically formed *de novo* in the tissue, and

subsequently, is mobilized in response to the energy demands of other tissues. This is similar to the dual function of the insect fat body (Newsholme and Start, 1973).

Visceral fat from suckers and goldfish in this study had relatively low or no NADPH generating activity (Tables 7, 8), and did not appear to be equivalent to rat epididymal fat pads or the fat body of frogs and insects in this regard. This finding seems to contradict the work of Farkas (1969), in which it was reported that the "adipose tissue" of bream, *Abramis brama*, and pike-perch, *Lucioperca lucioperca*, produced fatty acids. It is, in fact, not clear what tissue Farkas used, since personal observations by this author indicated no discrete lipogenic adipose tissue in suckers or goldfish, and only small amounts of visceral fat in pike, and this was not examined for enzyme activity. Aside from visceral fat, Vague and Fenasse (1965) indicate that suckers and goldfish have numerous subcutaneous adipocytes, and that pike may have considerable fat depots at the base of the fins. Neither of these depots were examined in the present work, and Vague and Fenasse (1965) indicate that they should not be considered as equivalent to mammalian adipose tissue.

The relatively low enzyme activity of the visceral fat of suckers and goldfish (Tables 7, 8) suggests that the visceral fat functions primarily as a depot of pre-formed lipid, although the αGPD activity of sucker visceral fat indicates that esterification of fatty acids may be taking place at this site. This activity, however, may be due to contamination of the homogenate with liver tissue. It is interesting to note that the measurable protein concentration in the supernatant fluid of sucker visceral fat homogenate is about one-half that of sucker liver (Table 5). With 200 μg/ml of protein present, one might expect some

enzyme activity in the visceral fat. Lipolytic activity in the form of lipases is probably present, in spite of the indirect demonstration by Farkas (1969) that no hormone-sensitive lipase existed in fish "adipose tissue." Unfortunately, this has not been investigated. In addition, to this author's knowledge, no examination has been made of the relationship between the comparatively diffuse liver of goldfish and suckers and the visceral fat, although changes in the two are of obvious importance on a seasonal basis.

Considering the low activity of the NADPH generating enzymes in the pike (Table 9), compared to other fish (Tables 7, 8, present work; Buhler and Benville, 1969; Braun et al., 1970; Nagayama et al., 1972; Shimeno and Takeda, 1972), it is probable that most of the lipid in the pike's body comes from its diet. The pike liver does have some lipogenic potential, however (Kluytmans and Zandee, 1974). The apparent absence of malic enzyme from the liver (Table 9) may be indicative of the generally low requirement for *de novo* lipogenesis, and may also indicate that glucose is not an important source of carbon for fatty acid synthesis (Hanson and Ballard, 1967). Most of the reducing equivalents produced via the pentose phosphate pathway are probably used for other reductive biosyntheses.

The white muscle tissue of pike, suckers, and goldfish showed low lipogenic potential in terms of G6PD, 6PGD, and ME activities (Tables 7-9). Considerable αGPD activity was present in the white muscle, however. This αGPD is probably not involved in the esterification of fatty acids or gluconeogenesis as in the liver (Harding et al., 1975), but is probably used to regenerate NAD^+ for triosephosphate dehydrogenase (Peterson et al., 1964), in conjunction with lactate dehydrogenase

during short hypoxic bursts of intensive muscular effort. The pike, in which such bursts are probably common, has higher αGPD activity in white muscle than do suckers or goldfish.

Among the $NADP^+$-dependent enzymes, isocitrate dehydrogenase (ICD) has probably received the most thorough attention from fish biochemists (Moon and Hochachka, 1971; Kunnemann and Passia, 1973). However, the exact physiological role of this enzyme remains to be established (Marr and Weber, 1969; Moon, 1972). A number of investigators (Pande et al., 1964; Wise and Ball, 1964; Leveille, 1970) have found no correlation or negative correlations between ICD activity and lipogenesis in rat adipose and liver tissue. For this reason, ICD was not included in the above discussion of lipogenesis. However, ICD activity is relatively high (Tables 7-9), and if fully expressed, would nearly double the NADPH production (Fig. 6) in goldfish and sucker livers, and quadruple the production in the pike liver.

Moon (1972) suggested that NADPH production for lipogenesis may be an important function of ICD, based on the significant inhibition of trout ICD by NADPH, and the findings of Flint and Denton (1970) that ICD was an important source of reducing equivalents for steroidogenesis in the rat ovary. However, the inhibition of pike and sucker G6PD by NADPH (Figs. 3A, B) occurs at lower $NADPH/NADP^+$ ratios than inhibition of trout ICD (Moon, 1972). This indicates, following the reasoning of Sanwal (1970), that G6PD is more tightly coupled to NADPH utilization by reductive biosyntheses, and, hence, G6PD is probably more important as a source of reducing equivalents for such processes.

B. Speculative Estimates of *in vivo* Lipogenesis

Although speculative, it is nonetheless interesting to attempt to estimate how much lipid can be produced by the liver of northern pike and white suckers at different times of year, based upon *in vitro* measurements of PP pathway dehydrogenases.

Figure 7 shows the estimates of liver lipid production at different temperatures for suckers and pike, and some of the assumptions required to make such estimates. This graph is obviously an extension of the more common graph expressing dehydrogenase activity as mol NADPH produced/min/gram (Fig. 6). Palmitate production is used as an index of lipid synthesis because it is quantitatively the most common saturated fatty acid formed *de novo* in many organisms (Masoro, 1968; Kluytmans and Zandee, 1974). The assumption that lipogenesis can stoichiometrically equal NADPH generation obviously ignores other avenues of NADPH utilization, which, on a percentage basis, may be important in the pike liver. In general, however, rates of NADPH production have been shown to closely parallel rates of fatty acid formation (Kather et al., 1972a). Expressing palmitate production estimates on a monthly basis is more realistic in terms of seasonal cycles than expressing it per minute as is usually done. The NADPH/NADP$^+$ ratio of 50 (from Greenbaum et al., 1971), used in Figure 7 is probably very close to the *in vivo* ratio, since it results in G6PD activity (calculated from the linear regression equations in Appendix IV) which is equal to or less than 6PGD activity (at 40 μM 6PG), a necessary condition if control is exerted at the G6PD reaction site (Greenbaum et al., 1971; Kather et al., 1972b). For suckers, G6PD is 35% of 6PGD activity at 22 C, and about 100% at 2 C. For pike, the respective percentages are 6% and 9%.

Figure 7. Estimates of *in vivo* palmitate production by white sucker (*Catostomus*) and northern pike (*Esox*) liver based upon *in vitro* pentose phosphate pathway dehydrogenase activity.

Estimates were calculated from the following assumptions and data:

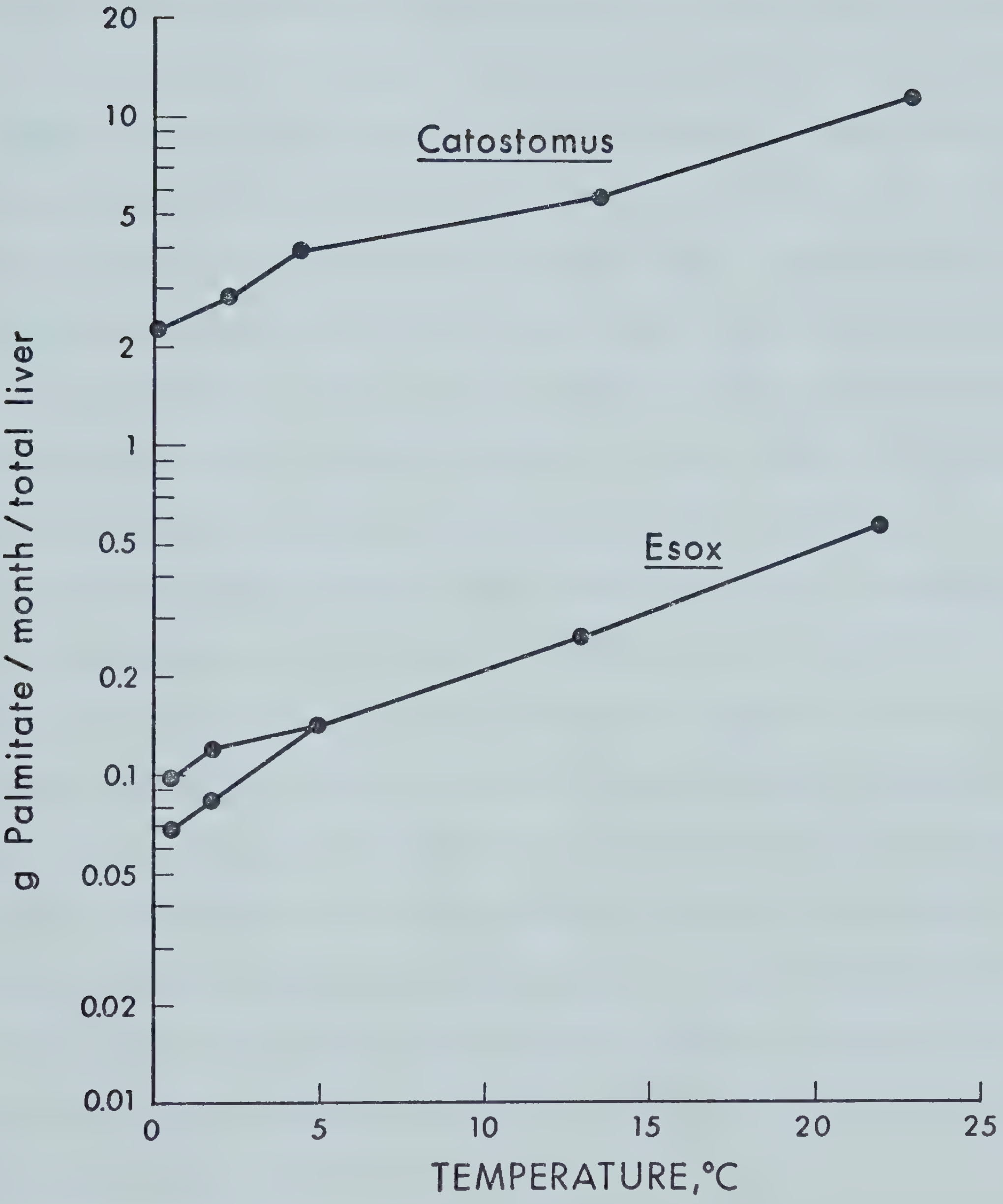
1. G6PD activity was calculated as in Figure 6. The G6P concentration was 0.2 mM (Appendix 5).

2. $NADPH/NADP^+ = 50$ (Greenbaum et al., 1971). The percentage activity from #1 (above) that occurred at this ratio was calculated from linear regression equations of the data presented in Figures 1A, B, Appendix IV.

3. The above estimates were doubled to include the NADPH produced by the 6PGD reaction.

4. Estimates of liver weight for northern pike were as in Figure 6. For white suckers, a 20 g liver (personal observations) was used in the calculations at all temperatures.

5. Micromoles NADPH produced/min/liver were converted to grams palmitate/month/liver based on the stoichiometric relationship 14 mmoles NADPH = 1 mmole palmitate (Masoro, 1968).


g Palmitate / month / total liver
20
10
5
2
1
0.5
0.2
0.1
0.05
0.02
0.01
Catostomus
Esox
0
5
10
15
20
25
TEMPERATURE,°C

The estimated amount of fatty acid synthesized *de novo* by the white sucker liver (Fig. 7) is about 6 to 12 grams per month during the summer, and 2 to 4 grams per month during the winter. The total lipid content of liver plus muscle is approximately 7 grams (Table 2, Appendix VI). It is estimated that the pike liver can synthesize (Fig. 7) no more than 0.6 gram of fatty acid per month during the summer, and less than 0.2 gram per month during the winter. However, the estimated total lipid content of pike liver plus muscle is about 4 grams. Comparing the fatty acid production estimates with the total lipid in the liver and muscle, one can suggest that, unless the lipid turnover rates are extremely low, it seems unlikely that *de novo* production of fatty acids by the liver is adequate to supply the lipids required, especially for the pike. Thus, one can conclude that preformed fatty acids from the diet almost certainly play an important role in the lipid economy of pike and probably suckers as well. This has previously been mentioned in regard to the pike (Kluytmans and Zandee, 1974). It would, therefore, be of interest to compare the total amount of lipid in the diet of the pike and suckers, and to see whether this information could be related to the above estimates of lipid production. Unfortunately, despite some knowledge about the diet of pike (Scott and Crossman, 1973), no quantitative estimates of lipid intake are available, and nothing is known about the total lipid in the sucker's diet.

It is obvious that there are a number of potential sources of error involved in both the estimates of fatty acid production via NADPH generation and in the interpretation of such estimates.

1. Conclusions about *in vivo* metabolic flux based upon enzyme studies can, at best, be considered as tentative; or as testable

hypotheses which must be correlated with other information (labelled precursor studies, substrate analysis, etc.; see Newsholme and Start, 1973). In some cases, estimates of *in vitro* G6PD and 6PGD activity do not appear to agree with estimates of carbon flow through the oxidative portion of the pentose phosphate pathway (Sapag-Hagar et al., 1973).

2. It is possible that the role of $NADP^+$-dependent isocitrate dehydrogenase should be given some consideration. As mentioned earlier, in mammals $NADP^+$-ICD does not act adaptively in concert with other lipogenic enzymes, so perhaps it forms a base-line of constant reducing equivalent production while the other enzymes exhibit a flexible response to dietary or hormonal stimuli. A similar function has been ascribed to malic enzyme in rat adipose tissue (Livrea et al., 1976).

3. Fatty acid production in the rest of the body (extra-hepatic lipogenesis) may be important enough to consider. Although it is probable that the liver is the most important organ of lipogenesis (Tables 7-9, present work; Braun et al., 1970; Kluytmans and Zandee, 1974), and that in suckers and goldfish, at least, visceral fat has relatively little or no lipogenic activity (Tables 7, 8), it is possible that the "adipose tissue" localized at the base of the fins in pike, and the subcutaneous adipocytes of cypriniformes (Vague and Fenasse, 1965) may contribute to fatty acid production. Unfortunately, not enough is known to make any estimates of extra-hepatic lipogenesis.

4. There is virtually no information on total lipid turnover rates in different organs of fish. Some information on the relative effect of temperature on these rates is available, however. It is known that proportionally both synthesis and breakdown can increase in cold-acclimated rainbow trout (Dean, 1969), and that, in goldfish, there is

differential synthesis and breakdown of individual fatty acids, and this is highly temperature-dependent (Knipprath and Mead, 1968).

In conclusion, rough estimates of *in vivo* lipogenesis from *in vitro* measurements of hepatic pentose phosphate pathway dehydrogenase activity indicate that white suckers can produce at least ten times more fatty acid than northern pike, and that preformed fatty acids from the diet may be an important source of body lipid in both species. Knowledge of the contributions of the diet, other enzymes, and other tissues to the overall lipid economy is inadequate, however. When the contributions of these factors are better known, particularly in relation to temperature, then the actual significance of possible proportional acclimatization-dependent increases in pentose phosphate pathway activity may be better understood.

LITERATURE CITED

Albers, C. 1970. Acid-base balance. *In* Fish Physiology (Edited by Hoar, W. S., and D. J. Randall), Vol. IV, pp. 173-208. Academic Press, New York.

Arese, P. 1964. Eine enzymatische Methode zur Bestimmung von 6-Phosphogluconat in biologischem Material. Biochem. Z. 340:345-350.

Atkins, G. L., and I. A. Nimmo. 1975. A comparison of seven methods for fitting the Michaelis-Menten equation. Biochem. J. 149:775-777.

Atkinson, D. E. 1967. Limitation of metabolite concentrations and the conservation of solvent capacity in the living cell. Curr. Topics Cell. Reg. 1:29-43.

Audilet, D. O., and I. Gray. 1973a. Temperature influences in developing tadpoles - I. Influence of temperature on the enzymatic activity of glucose-6-phosphate dehydrogenase. Cryobiology 10: 308-314.

Audilet, D. O., and I. Gray. 1973b. Temperature influences in developing tadpoles - II. Influence of temperature on isozymes and energies of activation of partially purified glucose-6-phosphate dehydrogenase. Cryobiology 10:315-320.

Avigad, G. 1966. Inhibition of glucose-6-phosphate dehydrogenase by adenosine-5-triphosphate. Proc. Nat. Acad. Sci. U.S.A. 56:1543-1547.

Baldwin, J., and P. W. Hochachka. 1970. Functional significance of isozymes in thermal acclimatization: acetylcholinesterase from trout brain. Biochem. J. 116:883-887.

Baranska, J., and Wlodawer. 1969. Influence of temperature on the composition of fatty acids and on lipogenesis in frog tissues. Comp. Biochem. Physiol. 28:553-570.

Behrisch, H. W. 1972. Molecular mechanisms of adaptation to low temperature in marine poikilotherms. Some regulatory properties of dehydrogenases from two arctic species. Mar. Biol. 13:267-275.

Bewley, G. C., J. M. Rawls, Jr., and J. C. Lucchesi. 1974. *Alpha*-glycerophosphate dehydrogenase in *Drosophila melanogaster*: kinetic differences and developmental differentiation of the larval and adult isozymes. J. Insect Physiol. 20:153-165.

Blazka, P. 1958. The anaerobic metabolism of fish. Physiol. Zool. 31: 117-128.

Bonsignore, A., and A. De Flora. 1972. Regulatory properties of glucose-6-phosphate dehydrogenase. Curr. Topics Cell. Reg. 6:21-62.

Bonsignore, A., I. Lorenzoni, R. Cancedda, and A. De Flora. 1970. Distinctive patterns of $NADP^+$ binding to dimeric and tetrameric glucose-6-phosphate dehydrogenase from human red cells. Biochem. Biophys. Res. Comm. 39:142-148.

Braun, K., H. Kunnemann, and H. Laudien. 1970. Der Einfluss von Temperaturänderungen auf Enzyme der Fischmuskulatur. Versuche mit *Rhodeus amarus*. Mar. Biol. 7:59-70.

Broyles, R. H., and C. F. Strittmatter. 1973. Hexose monophosphate shunt dehydrogenases in the developing frog. Comp. Biochem. Physiol. 44B:667-676.

Bücher, Th., K. Krejci, W. Russmann, H. Schnitger, and W. Wesemann. 1964. Metabolite assay in frozen samples of liver tissue. *In* Rapid Sampling Techniques in Biochemistry (Edited by Chance, B., R. W. Estabrook, and J. R. Williamson), pp. 255-264. Academic Press, New York.

Buhler, D. R., and P. Benville. 1969. Effect of feeding and of DDT on the activity of hepatic glucose-6-phosphate dehydrogenase in two salmonids. J. Fish. Res. Bd. Canada 26:3209-3216.

Chefurka, W. 1957. Oxidative metabolism of carbohydrates in insects - II. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase in the housefly, *Musca domestica*. L. Enzymologica 18: 209-227.

Cohen, P., and M. A. Rosemeyer. 1969. Human glucose-6-phosphate dehydrogenase: purification of the erythrocyte enzyme and the influence of ions on its activity. Eur. J. Biochem. 8:1-7.

Crowley, P. H. 1975. Natural selection and the Michaelis constant. J. Theor. Biol. 50:461-475.

Das, A. B. 1967. Biochemical changes in tissues of goldfish acclimated at high and low temperatures - II. Synthesis of protein and RNA of subcellular fractions and tissue composition. Comp. Biochem. Physiol. 21:469-485.

Dawson, R. M. C., D. C. Elliott, W. H. Elliott, and K. M. Jones. 1969. Data for Biochemical Research, p. 496. Oxford Univ. Press, Toronto.

Dean, J. M. 1969. The metabolism of tissues of thermally acclimated trout (*Salmo gairdneri*). Comp. Biochem. Physiol. 29:185-196.

Dixon, M. 1953. The determination of enzyme inhibitor constants. Biochem. J. 55:170-171.

Dixon, M., and E. C. Webb. 1964. Enzymes. Second edition. Academic Press, New York. 950 pp.

Dyson, J. E. D., and R. E. D'Orazio. 1971. 6-phosphogluconate dehydrogenase from sheep liver: inhibition of the catalytic activity by fructose-1,6-diphosphate. Biochem. Biophys. Res. Comm. 43:183-188.

Ekberg, D. R. 1958. Respiration in tissues of goldfish adapted to high and low temperatures. Biol. Bull. 114:308-316.

Ekberg, D. R. 1962. Anaerobic and aerobic metabolism in gills of the crucian carp adapted to high and low temperatures. Comp. Biochem. Physiol. 5:123-128.

Engel, P. C., and W. Ferdinand. 1973. The significance of abrupt transitions in Lineweaver-Burk plots with particular reference to glutamate dehydrogenase. Negative and positive co-operativity in catalytic rate constants. Biochem. J. 131:97-105.

Farkas, T. 1969. Studies on the mobilization of fats in lower vertebrates. Acta Biochem. Biophys. Acad. Sci. Hung. 4:237-249.

Flatt, J. P. 1970. Conversion of carbohydrate to fat in adipose tissue: an energy-yielding and, therefore, self-limiting process. J. Lipid Res. 11:131-143.

Flatt, J. P. and E. G. Ball. 1964. Studies on the metabolism of adipose tissue - XV. An evaluation of the major pathways of glucose catabolism as influenced by isnulin and epinephrine. J. Biol. Chem. 239: 675-685.

Flint, A. P. F., and R. M. Denton. 1970. The role of NADP-dependent malate dehydrogenase and isocitrate dehydrogenase in the supply of reduced NADP for steroidogenesis in the superovulated rat ovary. Biochem. J. 117:73-83.

Folch, J., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226:497-509.

Freed, J. M. 1971. Properties of muscle phosphofructokinase of cold- and warm-acclimated *Carassius auratus*. Comp. Biochem. Physiol. 39B: 747-764.

Fried, G. H., N. Greenberg, and W. Antopol. 1961. Determination of *alpha*-glycerophosphate oxidation with tetrazolium. Proc. Soc. Exp. Biol. Med. 107:523-525.

Gibson, D. M., R. T. Lyons, D. F. Scott, and Y. Muto. 1972. Synthesis and degradation of the lipogenic enzymes of rat liver. Adv. Enz. Reg. 10:187-204.

Glaser, L., and D. H. Brown. 1955. Purification and properties of D-glucose-6-phosphate dehydrogenase. J. Biol. Chem. 216:67-79.

Glock, G. E., and P. McLean. 1953. Further studies on the properties and assay of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase of rat liver. Biochem. J. 55:400-408.

Glock, G. E., and P. McLean. 1954. Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues and tumours. Biochem. J. 56:171-175.

Goodridge, A. G. 1973. Regulation of fatty acid synthesis in the liver of prenatal and early postnatal chicks. Hepatic concentrations of individual free fatty acids and other metabolites. J. Biol. Chem. 248:1939-1945.

Goodridge, A. G., and E. G. Ball. 1967. Lipogenesis in the pigeon: *in vivo* studies. Am. J. Physiol. 213:245-249.

Green, M. R., and B. R. Landau. 1965. Contribution of the pentose cycle to glucose metabolism in muscle. Arch. Biochem. Biophys. 111:569-575.

Greenbaum, A. L., K. A. Gumaa, and P. McLean. 1971. The distribution of hepatic metabolites and the control of the pathways of carbohydrate metabolism in animals of different dietary and hormonal status. Arch. Biochem. Biophys. 143:617-663.

Hanson, R. W., and F. J. Ballard. 1967. The relative significance of acetate and glucose as precursors for lipid synthesis in liver and adipose tissue from ruminants. Biochem. J. 105:529-536.

Harding, J. W. Jr., E. A. Pyeritz, H. P. Morris, and H. B. White, III. 1975. Proportional activities of glycerol kinase and glycerol 3-phosphate dehydrogenase in rat hepatomas. Biochem. J. 148:545-550.

Hazel, J. R., and C. L. Prosser. 1974. Molecular mechanisms of temperature compensation in poikilotherms. Physiol. Rev. 54:620-677.

Hochachka, P. W. 1967. Organization of metabolism during temperature compensation. *In* Molecular Mechanisms of Temperature Adaptation (Edited by Prosser, C. L.), pp. 177-203. AAAS publication number 84, Washington, D.C.

Hochachka, P. W., and F. R. Hayes. 1962. The effect of temperature acclimation on pathways of glucose metabolism in the trout. Can. J. Zool. 40:261-270.

Hochachka, P. W., and B. C. Hochachka. 1973. Glucose-6-phosphate dehydrogenase and thermal acclimation in the mullet fish. Mar. Biol. 18:251-259.

Hochachka, P. W., and G. N. Somero. 1971. Biochemical adaptation to the environment. *In* Fish Physiology (Edited by Hoar, W. S., and D. J. Randall), Vol. VI, pp. 99-156. Academic Press, New York.

Hochachka, P. W., and G. N. Somero. 1973. Strategies of Biochemical Adaptation. W. B. Saunders Co., Philadelphia. 358 pp.

Hochachka, P. W., G. N. Somero, D. E. Schneider, and J. M. Freed. 1970. The organization and control of metabolism in the crustacean gill. Comp. Biochem. Physiol. 33:529-548.

Hogeboom, G. H. 1955. Fractionation of cell components of animal tissues. *In* Methods in Enzymology (Edited by Colowick, S. P., and N. O. Kaplan), Vol. I, pp. 16-19. Academic Press, New York.

Horecker, B. L., and P. Z. Smyrniotis. 1955. 6-phosphogluconic dehydrogenase. *In* Methods in Enzymology (Edited by Colowick, S. P., and N. O. Kaplan), Vol. I, pp. 323-327. Academic Press, New York.

Ince, B. W., and A. Thorpe. 1976. The effects of starvation and force-feeding on the metabolism of the northern pike, *Esox lucius* L. J. Fish. Biol. 8:79-88.

Ingle, D. L., D. E. Bauman, and U. S. Garrigus. 1972. Lipogenesis in the ruminant: *in vitro* study of tissue sites, carbon source, and reducing equivalent generation for fatty acid synthesis. J. Nutr. 102: 609-616.

Inui, Y., and Y. Ohshima. 1966. Effect of starvation on metabolism and chemical composition of eels. Bull. Jap. Soc. Scient. Fish. 32: 492-501.

Jansen, G. R., C. F. Hutchinson, and M. E. Zanetti. 1966. Studies on lipogenesis *in vivo*. Effect of dietary fat or starvation on conversion of (^{14}C)glucose into fat and turnover of newly synthesized fat. Biochem. J. 99:323-332.

Johnston, I. A., and G. Goldspink. 1973. A study of the swimming performance of the Crucian carp *Carassius carassius* (L.) in relation to the effects of exercise and recovery on biochemical changes in the myotomal muscles and liver. J. Fish. Biol. 5:249-260.

Kamada, T., and S. H. Hori. 1970. A phylogenetic study of animal glucose-6-phosphate dehydrogenases. Jap. J. Genetics 45:319-339.

Kanungo, M. S., and C. L. Prosser. 1959. Physiological and biochemical adaptation of goldfish to cold and warm temperatures - II. Oxygen consumption of liver homogenate; oxygen consumption and oxidative phosphorylation of liver mitochondria. J. Cell. Comp. Physiol. 54: 265-274.

Kather, H., M. Rivera, and K. Brand. 1972a. Interrelationship and control of glucose metabolism and lipogenesis in isolated fat cells. Effect of the amount of glucose uptake on the rates of the pentose phosphate cycle and of fatty acid synthesis. Biochem. J. 128: 1089-1096.

Kather, H., M. Rivera, and K. Brand. 1972b. Interrelationship and control of glucose metabolism and lipogenesis in isolated fat cells. Control of pentose phosphate-cycle activity by cellular requirement for reduced NADP. Biochem. J. 128:1097-1102.

Katz, J., and R. Rognstad. 1966. The metabolism of tritiated glucose by rat adipose tissue. J. Biol. Chem. 241:3600-3610.

Kluytmans, J. H. F. M., and D. I. Zandee. 1974. Lipid metabolism in the northern pike (*Esox lucius* L.) - 3. *In vivo* incorporation of 1-^{14}C-acetate in the lipids. Comp. Biochem. Physiol. 48B:641-649.

Knipprath, W. G., and J. F. Mead. 1968. The effect of the environmental temperature on the fatty acid comoosition and on the *in vivo* incorporation of 1-^{14}C-acetate in goldfish (*Carassius auratus* L.). Lipids 3:121-128.

Kornberg, A., and B. L. Horecker. 1955. Glucose-6-phosphate dehydrogenase. *In* Methods in Enzymology (Edited by Colowick, S. P., and N. O. Kaplan), Vol. I, pp. 323-327. Academic Press, New York.

Krebs, H. A., and R. L. Veech. 1969. Equilibrium relations between pyridine nucleotides and adenine nucleotides and their roles in the regulation of metabolic processes. Adv. Enz. Reg. 7:397-413.

Künnemann, H., H. Laudien, and H. Precht. 1970. Der Einfluss von Temperaturänderungen auf Enzyme der Fischmuskulatur. Versuche mit Goldorfen *Idus idus*. Mar. Biol. 7:71-81.

Künnemann, H., and D. Passia. 1973. NADP^{+}-Isocitrat-Dehydrogenase aus *Idus idus* (Pisces: Cyprinidae). II. Einfluss der Temperatur auf Substrat- und Cosubstrataffinität. Mar. Biol. 23:205-211.

Langer, O. E. 1974. Seasonal variation in food, mouth anatomy, and distribution of adult yellow perch (*Perca fluviatilis flavescens*) and yellow walleye (*Stizostedion vitreum vitreum*) in Lac Ste Anne, M.Sc. Thesis. Univ. of Alberta, Edmonton, Alberta. 119 pp.

Lehmann, J. 1970. Veränderungen der Enzymaktivitäten nach einem Wechsel der Adaptationstemperatur, untersucht am Seitenrumpfmuskel des Goldfisches (*Carassius auratus* L.). Int. Revue Ges. Hydrobiol. 55: 763-781.

Leveille, G. A. 1970. Adipose tissue metabolism: influence of periodicity of eating and diet composition. Fed. Proc. 29:1294-1301.

Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56:658-666.

Livrea, G., G. Carrozza, L. Manasseri, and U. Muraca. 1976. Concomitant repression by gluten of HMP shunt dehydrogenases, citrate cleavage enzyme, and malic enzyme in rat liver and adipose tissue. J. Nutr. 106:335-341.

Love, R. M. 1970. The Chemical Biology of Fishes. Academic Press, New York. 547 pp.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

Mackay, W. C., and D. D. Beatty. 1968. Plasma glucose levels of the white sucker, *Catostomus commersonii*, and the northern pike, *Esox lucius*. Can. J. Zool. 46:797-803.

Manohar, S. V. 1970. Postmortem glycolytic and other biochemical changes in white muscle of white sucker (*Catostomus commersoni*) and northern pike (*Esox lucius*) at 0 C. J. Fish. Res. Bd. Canada 27:1997-2002.

Marr, J. J., and M. M. Weber. 1969. Concerted inhibition of a $NADP^+$-specific isocitrate dehydrogenase and the implications for metabolic regulation. Biochem. Biophys. Res. Commun. 35:12-19.

Maslennikova, N. V. 1970. Seasonal changes in the nutritional value of Baltic cod. Tr. Vses. Naucho-Issled. Inst. Morsk. Rybn, Khoz. Okeanogr. (VNIRO) 73:73-86. (Transl. from Russian by Fish. Res. Bd. Canada Transl. Ser. No. 1731, 1971.)

Masoro, E. J. 1968. Physiological Chemistry of Lipids in Mammals. W. B. Saunders Company, Toronto. 304 pp.

Massey, V., B. Curti, and H. Ganther. 1966. A temperature-dependent conformational change in D-amino acid oxidase and its effect on catalysis. J. Biol. Chem. 241:2347-2357.

Medford, B. A. 1976. Annual cycle in condition and nutrient composition of northern pike (*Esox lucius* L.). M.Sc. Thesis. Univ. of Alberta, Edmonton, Alberta. 61 pp.

Mester, R., D. Scripcariu, and S. Niculescu. 1972. Effect of temperature on the isoenzyme pattern of loach (*Misgurnus fossilis* L.) I. Glucose-6-phosphate dehydrogenase, NAD- and NADP-isocitrate dehydrogenase. Rev. Roum. Biol.-Zool. 17:205-217. (Cited by Moon, T. W., 1975. Temperature adaptation: isozymic function and the maintenance of heterogeneity. *In* Isozymes II: pp. 207-220. Academic Press, New York.)

Michaelis, O. E., and B. Szepesi. 1973. Effect of various sugars on hepatic glucose-6-phosphate dehydrogenase, malic enzyme, and total liver lipid of the rat. J. Nutr. 103:697-705.

Moon, T. W. 1972. The functional adaptation of enzymes to temperature: comparison of NADP-linked isocitrate dehydrogenases of trout liver and pig heart. Comp. Biochem. Physiol. 43B:525-538.

Moon, T. W., and P. W. Hochachka. 1971. Temperature and enzyme activity in poikilotherms: isocitrate dehydrogenases in rainbow-trout liver. Biochem. J. 123:695-705.

Moroz, I. Ye. 1971. Dynamics of metabolism in the carp (*Cyprinus carpio* L.) during overwintering. J. Ichthyol. 11:592-595.

Muto, S., and I. Uritani. 1972. Glucose-6-phosphate dehydrogenase from sweet potato: effect of various ions and their ionic strength on enzyme activity. Plant and Cell Physiol. 13:111-118.

Nagayama, F., and H. Ohshima. 1975. Studies on the enzyme system of carbohydrate metabolism in fish - IV. Properties of phosphogluconate dehydrogenase. Bull. Jap. Soc. Scient. Fish. 41:1125-1128.

Nagayama, F., H. Ohshima, and T. Takeuchi. 1975a. Studies on the enzyme system of carbohydrate metabolism in fish - II. Purification of glucose-6-phosphate dehydrogenase and glucose dehydrogenase. Bull. Jap. Soc. Scient. Fish. 41:1063-1067.

Nagayama, F., H. Ohshima, and T. Takeuchi. 1975b. Studies on the enzyme system of carbohydrate metabolism in fish - III. Properties of glucose-6-phosphate dehydrogenase and glucose dehydrogenase. Bull. Jap. Soc. Scient. Fish. 41:1069-1074.

Nagayama, F., H. Ohshima, and K. Umezawa. 1972. Distribution of glucose-6-phosphate metabolizing enzymes in fish. Bull. Jap. Soc. Scient. Fish. 38:589-593.

Newsholme, E. A., and C. Start. 1973. Regulation in Metabolism. John Wiley and Sons, Toronto. 349 pp.

O'Hea, E. K., and G. A. Leveille. 1969a. Lipid biosynthesis and transport in the domestic chick (*Gallus domesticus*). Comp. Biochem. Physiol. 30:149-159.

O'Hea, E. K., and G. A. Leveille. 1969b. Significance of adipose tissue and liver as sites of fatty acid synthesis in the pig and the efficiency of utilization of various substrates for lipogenesis. J. Nutr. 99:338-344.

Pande, S. V., R. P. Kahn, and T. A. Venkitasubramanian. 1964. Nicotinamide adenine dinucleotide phosphate-specific dehydrogenases in relation to lipogenesis. Biochem. Biophys. Acta 84:239-250.

Passonneau, J. V., D. W. Schultz, and O. H. Lowry. 1966. The kinetics of glucose-6-phosphate dehydrogenase. Fed. Proc. 25:219. Abst.

Precht, H., J. Christopherson, H. Hensel, and W. Larcher. 1973. Temperature and Life, p. 330. Springer-Verlag, New York.

Prosser, C. L., W. Mackay, and K. Kato. 1970. Osmotic and ionic concentrations in some Alaskan fish and goldfish from different temperatures. Physiol. Zool. 43:81-89.

Rahn, H. 1966. Gas transport from the external environment to the cell. *In* Development of the Lung (Edited by Reuck, A. V. S. de, and R. Porter), pp. 3-23. J. A. Churchill Ltd., London.

Rahn, H., and F. W. Baumgardner. 1972. Temperature and acid-base regulation in fish. Resp. Physiol. 14:171-182.

Ringler, M. I., and R. Hilf. 1974. Kinetics of glucose-6-phosphate dehydrogenase from normal and neoplastic mammary tissue. Enzyme 18:257-261.

Rimsh, E. Ya. 1970. Some peculiarities of protein and lipid metabolism in grass carp. Tr. Vses. Naucho-Issled. Inst. Morsk. Rybn. Khoz. Okeanogr. (VNIRO) 74:222-243. (Transl. from Russian by Fish. Res. Bd. Canada Trans. Ser. No. 1770, 1971.)

Robert, M., and I. Gray. 1972a. Enzymatic mechanisms during temperature acclimation of the blue crab, *Callinectes sapidus* - I. Oxygen consumption and activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Comp. Biochem. Physiol. 42:377-387.

Robert, M., and I. Gray. 1972b. Enzymatic mechanisms during temperature acclimation of the blue crab, *Callinectes sapidus* - II. Kinetic and thermodynamic studies of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. Comp. Biochem. Physiol. 42B: 389-402.

Rutter, W. J. 1957. The effect of ions on the catalytic activity of enzymes: yeast glucose-6-phosphate dehydrogenase. Acta Chem. Scand. 11:1576-1586.

Sacktor, B., and E. Wormser-Shavit. 1966. Regulation of metabolism in working muscle *in vivo* - I. Concentrations of some glycolytic, tricarboxylic acid cycle, and amino acid intermediates in insect flight muscles during flight. J. Biol. Chem. 241:624-631.

Sanwal, B. D. 1970. Regulatory mechanisms involving nicotinamide adenine nucleotides as allosteric effectors - III. Control of glucose-6-phosphate dehydrogenase. J. Biol. Chem. 245:1626-1631.

Sapag-Hagar, M., R. Lagunas, and A. Sols. 1973. Apparent unbalance between the activities of 6-phosphogluconate and glucose-6-phosphate dehydrogenases in rat liver. Biochem. Biophys. Res. Commun. 50: 179-185.

Scott, W. B., and E. J. Crossman. 1973. Freshwater Fishes of Canada. Fish. Res. Bd. Canada, Bull. 184. Ottawa. 966 pp.

Seifter, S., S. Dayton, B. Novic, and E. Muntwyler. 1950. The estimation of glycogen with the anthrone reagent. Arch. Biochem. Biophys. 25:191-200.

Shimeo, S., and M. Takeda. 1972. Studies on hexose monophosphate shunt of fishes - I. Properties of hepatic glucose-6-phosphate dehydrogenase of barracuda. Bull. Jap. Soc. Scient. Fish. 38:645-650.

Shrago, E., J. A. Glennon and E. S. Gordon. 1971. Comparative aspects of lipogenesis in mammalian tissues. Metabolism 20:54-61.

Shul'man, G. E. 1974. Life Cycles of Fish. John Wiley and Sons, Toronto. 258 pp.

Somero, G. N. 1969. Enzymic mechanisms of temperature compensation: immediate and evolutionary effects of temperature on enzymes of aquatic poikilotherms. Amer. Natur. 103:517-530.

Somero, G. N. 1975a. The roles of isozymes in adaptation to varying temperatures. *In* Isozymes II: Physiology and Function (Edited by Markert, C. L.), pp. 221-234. Academic Press, New York.

Somero, G. N. 1975b. Temperature as a selective factor in protein evolution: the adaptational strategy of "compromise". J. Exp. Zool. 194:175-188.

Stimpson, J. H. 1965. Comparative aspects of the control of glycogen utilization in vertebrate liver. Comp. Biochem. Physiol. 15:187-197.

Tashima, L. S., and G. F. Cahill, Jr. 1965. Fat metabolism in fish. *In* Handbook of Physiology, Section 5, Adipose Tissue (Edited by Renold, A. E., and G. F. Cahill, Jr.), pp. 55-58. Amer. Physiol. Soc., Washington, D.C.

Vague, J., and R. Fenasse. 1965. Comparative anatomy of adipose tissue. *In* Handbook of Physiology, Section 5, Adipose Tissue (Edited by Renold, A. E., and G. F. Cahill, Jr.), pp. 25-36. Amer. Physiol. Soc., Washington, D.C.

Van Handel, E. 1965. Estimation of glycogen in small amounts of tissue. Analyt. Biochem. 11:256-265.

Vroman, H. E., and J. R. C. Brown. 1963. Effect of temperature on the activity of succinic dehydrogenase from the livers of rats and frogs. J. Cellular Comp. Physiol. 61:129-131.

Wilkinson, G. N. 1961. Statistical estimations in enzyme kinetics. Biochem. J. 80:324-332.

Wilson, F. R., G. S. Whitt, and C. L. Prosser. 1973. Lactate dehydrogenase and malate dehydrogenate isozyme patterns in tissues of temperature-acclimated goldfish (*Carassius auratus* L.). Comp. Biochem. Physiol. 46B:105-116.

Wise, E. M., Jr., and E. G. Ball. 1964. Malic enzyme and lipogenesis. Proc. Nat. Acad. Sci. USA 52:1255-1263.

Yoshida, A. 1966. Glucose-6-phosphate dehydrogenase of human erythrocytes - I. Purification and characterization of normal (B^+) enzyme. J. Biol. Chem. 241:4966-4976.

Yoshida, A. 1973. Hemolytic anemia and G6PD deficiency. Science 179: 532-537.

Addenda:

Bonitati, J., W. B. Elliott, and P. G. Miles. 1969. Interference by carbohydrate and other substances in the estimation of protein with the Folin-Ciocalteu reagent. Analyt. Biochem. 31:399-404.

Gellert, M. F., P. H. von Hippel, H. K. Schachman, and M. F. Morales. 1959. Studies on the contractile proteins of muscle. I. The ATP-myosin B interaction. J. Amer. Chem. Soc. 81:1384-1393.

Gerhardt, B., and H. Beevers. 1968. Influence of sucrose on protein determination by the Lowry procedure. Analyt. Biochem. 24:337-339.

Peterson, R. D., D. Gaudin, R. M. Bocek, and C. H. Beatty. 1964. a-Glycerophosphate metabolism in muscle under aerobic and hypoxic conditions. Amer. J. Physiol. 206:599-602.

APPENDIX I. pH, Ions, and Bimodal Kinetics

A. Results

The activity of goldfish G6PD and 6PGD in the presence of different hydrogen ion concentrations (pH) is shown in Figures 1A and 1B. G6PD exhibited maximal or near-maximal activity at pH 8.0 to 8.8 (Fig. 1A). 6PGD had a somewhat narrower optimum around pH 8.0 (Fig. 1B).

The Tris-HCl buffer used in the pH optima studies had a constant molarity, but varied in ionic strength. Increases in ionic strength above 0.07 M decreased the activity of G6PD (Fig. 2), and presumably 6PGD, as well (Table 1). The ionic strength of the buffer (pH 8.0) routinely used was 0.064 M. The ionic strength of the homogenate in the assay cuvette probably did not exceed 0.01 M because of the large dilution factor, and so the ionic strength of the homogenate will be ignored in the following discussion.

Magnesium ions inhibit G6PD activity at concentrations greater than 2 mM (Fig. 2). Similar effects were seen with 6PGD (Table 1). A comparison of the effects of Mg^{++} and Tris-HCl buffer in terms of ionic strength is shown in Figure 2.

The inhibitory effects of mercuric ions (Table 1) are common in studies of G6PD and 6PGD (Broyles and Strittmatter, 1973), and are not due to changes in ionic strength, since the $HgCl_2$ concentrations are too low to significantly change this parameter.

Some of the double-reciprocal plots of liver G6PD activity (Appendix III) showed a trend toward bimodality at higher substrate concentrations. This is particularly pronounced in the plot of northern

Figure 1A (Upper). The effect of pH on the activity of goldfish (*Carassius*) liver G6PD. The activity at pH 8.0 was considered to be the control. The assay solution contained saturating amounts of G6P (1.0 mM) and $NADP^+$ (0.4 mM). Tris-HCl buffer (100 mM) was used to adjust the pH of the assay solution. The assay temperature was 22.0 C.

(●) 20 C acclimated fish

(o) 2 C acclimated fish

Figure 1B (Lower). The effect of pH on the activity of goldfish (*Carassius*) liver 6PGD. The assay solution contained saturating amounts of 6PG (1.0 mM) and $NADP^+$ (0.3 mM). Other details as in Fig. 1A.

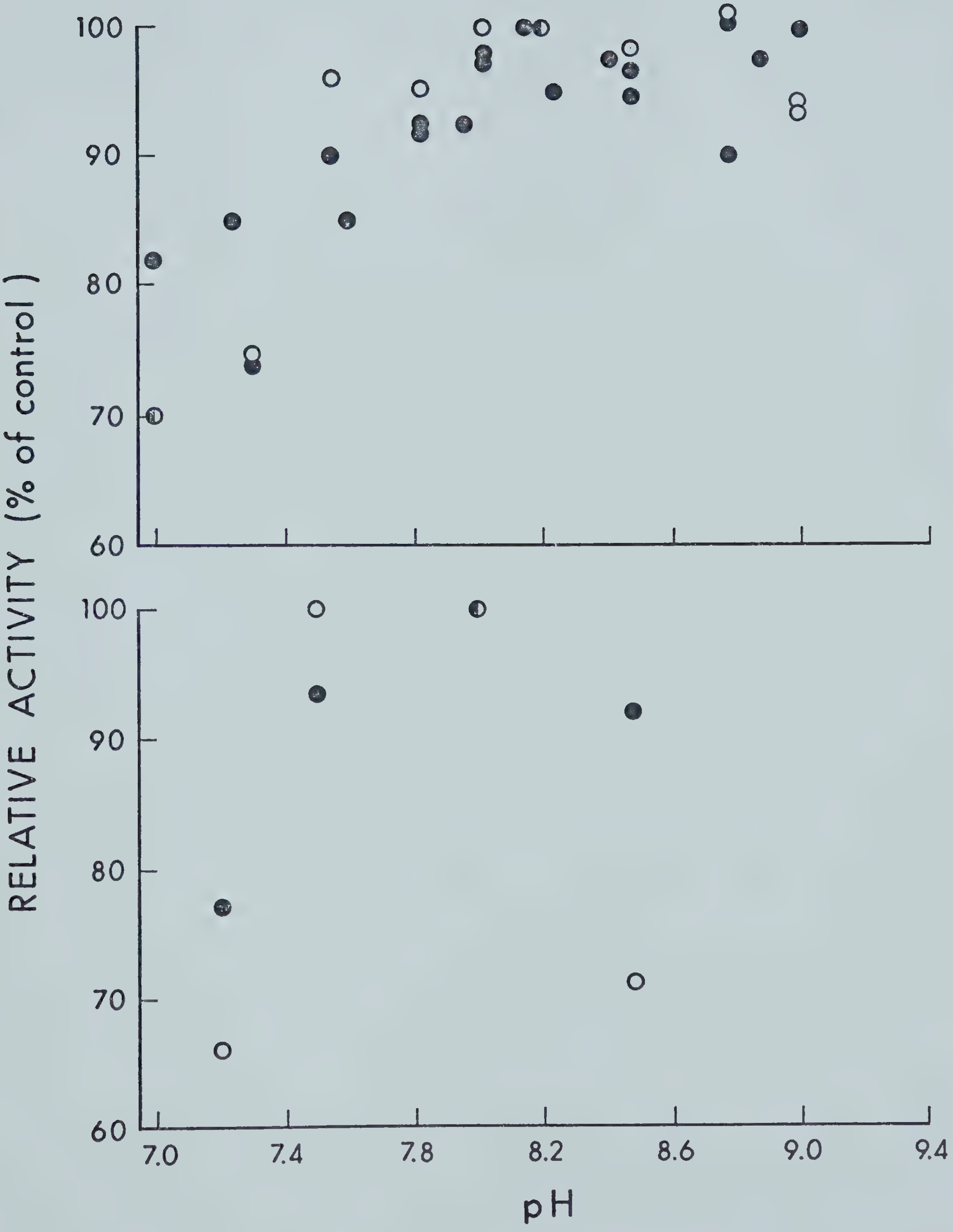
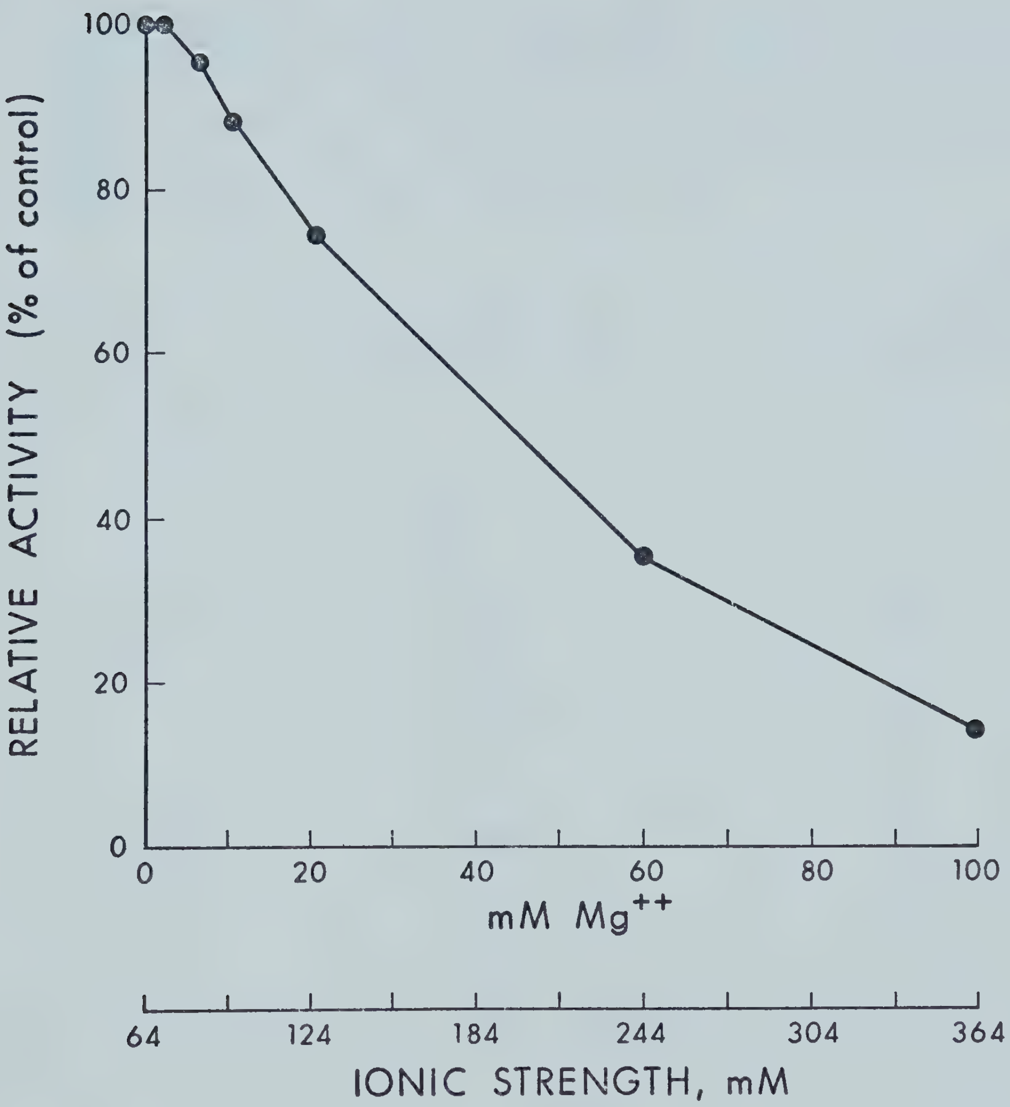

RELATIVE ACTIVITY (% of control)
100
90
80
70
60
100
90
80
70
60
7.0
7.4
7.8
8.2
8.6
9.0
9.4
pH

Figure 2. The effect of Mg^{++} (as $MgCl_2$) and ionic strength on the activity of goldfish (*Carassius*) liver G6PD. The G6P concentration was 0.1 mM; $NADP^+$ was saturating; and the concentration of Tris-HCl buffer was 100 mM (ionic strength, 64 mM). The control cuvette contained no Mg^{++}. The assay temperature was 22.0 C.


RELATIVE ACTIVITY (% of control)
100
80
60
40
20
0
0
20
40
60
80
100
mM Mg^{++}
64
124
184
244
304
364
IONIC STRENGTH, mM

APPENDIX I (Continued)

Table 1. The effect of Mg^{++} and Hg^{++} on goldfish (*Carassius*) liver 6PGD and G6PD activity. In both cases, chloride salts were used, and the concentrations represent the final concentrations in the assay medium. The G6P concentration was 0.1 mM; $NADP^{+}$ was saturating. No correction was made for the EDTA present in the initial homogenizing fluid. The assay temperature was 22 C.

Enzyme	Ion	Concentration (mM)	% Relative activity
6PGD	Mg^{++}	0.0	100
"	"	10.0	89
"	"	100.0	10
6PGD	Hg^{++}	0.000	100
"	"	0.001	100
"	"	0.01	19
G6PD	Hg^{++}	0.000	100
"	"	0.001	100
"	"	0.01	21

pike G6PD (Fig. 2, Appendix III). At an assay temperature of 11.3 C, for example, using only the substrate concentrations below 0.5 mM, the Km(G6P) was 44 μM and the Vmax was 405 nmol NADPH produced/min/gram liver. Using substrate concentrations of 0.5 mM and above, the Km(G6P) was 228 μM and the Vmax was 530 nmol NADPH produced/min/gram liver (Fig. 2, Appendix III).

B. Discussion

Modulation of the activity of poikilotherm enzymes may not only occur due to changes in the concentrations of effector metabolites or temperature, but may also be due to changes in other factors in the intracellular milieu, such as pH, concentration of various ions, and ionic strength (Hochachka and Somero, 1973).

The combined effects of hydrogen ion concentration and ionic strength resulted in a pH optimum for goldfish liver G6PD (Fig. 1A) of approximately 8.0 to 8.8, which is identical to optima reported for G6PD enzymes from a number of organisms (for example, yeast G6PD, Glaser and Brown, 1955; human erythrocyte G6PD, Yoshida, 1966; and G6PD from four teleost species, Nagayama et al., 1975b). Cohen and Rosemeyer (1969) have shown that, with human erythrocyte G6PD, this pH optimum is found only if buffers of constant molarity and varying ionic strength are used. With constant ionic strength buffers, they found that the activity increased with pH, up to at least pH 9.0.

In poikilotherms, Rahn (1966) and co-workers (Rahn and Baumgardner, 1972) have shown that blood pH (and perhaps intracellular pH) increases with decreasing temperature to maintain a level approximately 0.5 units above the pN. G6PD activity *in vivo* may thus increase as the pH

increases with lowering temperature, similar to the situation found by Freed (1971) for goldfish muscle phosphofructokinase.

The pH optimum for 6PGD (pH 8.0, Fig. 1B) is similar to those optima reported elsewhere for carp, rainbow trout, and eel 6PGD (Nagayama and Ohshimi, 1975), but there is considerable variability in the literature. For example, Glock and McLean (1954), using rat liver 6PGD, found a pH optimum of 9.0.

Cohen and Rosemeyer (1969) have demonstrated that both the stimulatory and inhibitory effects of Mg^{++} on human erythrocyte G6PD were due primarily to an increase in ionic strength. At low concentrations of Mg^{++}, the Vmax is increased, but at higher concentrations (>0.05 M), Mg^{++} competes with G6P for binding sites (see also Rutter, 1957), and can severely inhibit the reaction by greatly increasing the Km(G6P). The inhibition of goldfish liver G6PD and 6PGD by Mg^{++} at concentrations above 2 mM is probably due, at least in part, to the increasing ionic strength, as well, as shown by the inhibition caused by 0.2 M Tris-HCl buffer (Fig. 2). The response of the unpurified goldfish liver G6PD (Fig. 2) was similar to the response of the highly purified human erythrocyte G6PD (Fig. 6 in Cohen and Rosemeyer, 1969), except that the goldfish enzyme appeared to be somewhat more sensitive to inhibition by high ionic strength. Gill G6PD from the tanner crab, *Chionocetes bairdi* (Behrisch, 1972) appeared to be much less sensitive to inhibition by ionic strength than either the goldfish liver or human erythrocyte enzymes, and this may reflect the higher ionic strength of its environment.

The effects of ionic strength, as distinct from the effects of different ions, have not been investigated in 6PGD, although inhibition

by some ions has been demonstrated (Table 1, this work; Chefurka, 1957).

Intracellular ionic strength is probably considerably higher than 0.1 M (Albers, 1970, p. 185), and this suggests that the ionic strength of the intracellular medium probably contributes to the partial inhibition of G6PD and 6PGD *in vivo*. Changes in ionic strength due to the interactions of temperature and pH, or due to a decrease in ion content in the tissues at lower temperatures (Prosser et al., 1970), could, therefore, result in changes in enzyme activity *in vivo*. For instance, a decrease in ionic strength of 0.01 M from 0.16 M to 0.15 M could increase G6PD activity by about 5% (Fig. 2). It is important to realize, however, that different enzymes are affected differently by pH, ionic strength, and specific ions, and under conditions that increase the activities of some enzymes, other enzyme activities may decrease significantly. Thus, the usefulness of this type of modulation in rate-compensation may be limited (Hochachka and Somero, 1973).

Substrate activation of G6PD by high concentrations of G6P (Fig. 2, Appendix III) has been explained as negative cooperativity by some workers (Muto and Uritani, 1972; Engel and Ferdinand, 1973; Ringler and Hilf, 1974). Based upon the similarities between the activation data presented by Muto and Uritani (1972), Ringler and Hilf (1974), and Figure 2 in Appendix III, this explanation may well be the correct one, although no further evidence of subunit interaction was found in Figure 2, Appendix III, such as the cooperativity found at low $NADP^+$ concentrations in text Figures 2A and 2B. Muto and Uritani (1972) have shown that high G6P concentrations can cause dissociation of G6PD into subunits, with concomitant changes in both Km and Vmax. The magnitude of the effects

decreased with increasing ionic strength. It is also possible that the substrate activation of G6PD may be explained by assuming that the higher concentrations of G6P are overcoming the competitive inhibition of G6PD by Mg^{++} (Cohen and Rosemeyer, 1969). It is probable that this is not the complete explanation, since the Vmax (y-intercept, Fig. 2, Appendix III) changes, and this would probably not occur if G6PD were competitively inhibited. It is possible that a combination of subunit interactions and/or dissociations and ion inhibition may be involved.

It is interesting to observe that, of the three G6PD enzymes examined, northern pike liver G6PD, with the lowest activity, the lowest Km(G6P), and most temperature insensitive apparent enzyme-substrate affinity at high to moderately low temperatures (22-5 C), was the only G6PD which consistently showed substrate activation. Since high concentrations (>0.2 mM) of G6P may be found in the cell (Appendix V), the substrate activation of G6PD can be potentially important *in vivo*.

APPENDIX II. A Comparison of the Single and Double Substrate Procedures for the Measurement of Pentose Phosphate Pathway Dehydrogenase Activity

Several, slightly different assay procedures have been used for the measurement of the activity of pentose phosphate pathway dehydrogenases. The assay which directly measures the reduction of $NADP^+$ at 340 nm is essentially that of Glock and McLean (1953) and Kornberg and Horecker (1955), and has been used, with minor modifications, by nearly all later investigators. Glock and McLean (1953) used three different procedures to measure the activity of glucose-6-phosphate dehydrogenase. The single substrate procedure, in which glucose-6-phosphate (G6P) is the only substrate present when measuring G6PD activity, is used in the present study. Another procedure uses only G6P as the substrate, but a high concentration of 6-phosphogluconate dehydrogenase is added to completely convert the G6P to a pentose sugar. One-half of the activity is then due to G6PD. The third variation (the double substrate procedure), and one commonly used (Flatt, 1970; Robert and Gray, 1972a), involves adding equimolar amounts of G6P and 6PG in one cuvette, and only 6PG in a second cuvette. The activity of the latter is subtracted from the activity of the former to obtain G6PD activity.

A comparison of the single and double substrate procedures indicated that the G6PD activity as estimated by the single substrate method was 7 to 46% greater than that obtained using the double substrate method (Table 1). However, with the exception of assays which last for considerable periods of time (for example, Michaelis and Szepesi, 1973),

APPENDIX II (Continued)

Table 1. Some comparisons of the estimates of G6PD activity using the single and double substrate procedures of Glock and McLean (1953). Velocity is expressed as nmol NADPH produced/min/gram wet weight of tissue. Equimolar concentrations (0.1 mM) of G6P and 6PG were used.

	G6PD activity	
Species and tissue	Single substrate (G6P)	Double substrate (G6P, 6PG)
Carassius, liver	4,100	3,135
Esox, liver	530	386
Catostomus, brain	424	290
Catostomus, gill	617	579

the amount of 6PG produced by the G6PD reaction in a 3 to 5 minute assay period is far too low to cause any appreciable error from 6PGD activity. Some simple calculations will illustrate this point. The greatest G6PD activity measured in this investigation was approximately 0.100 μmol NADPH produced per minute in the assay cuvette. (Normally the activity was less than 0.03 μmol per min.) This corresponds to 0.300 μmol 6PG present in the cuvette at the end of a 3 minute assay. Activity of G6PD measured by Glock and McLean (1954) did not exceed 0.015 μmol per minute or a total of 0.075 μmol 6PG produced in a 5 minute assay. At either of these concentrations of substrate, the 6PGD reaction, *in vitro*, was virtually inactive, since enzyme activity is, in simplest terms, dependent upon substrate concentration. The 6PGD reaction, as extrapolated from regression equations of substrate-dependent 6PGD activity (Appendix III) could not possibly account for more than 1 or 2% of the total activity measured.

The reason for the sometimes large disagreement (Table 1) between the two procedures is not entirely clear, but it may be due to product inhibition of G6PD by the 6PG added in the double substrate procedure. This possibility has not, to my knowledge, been investigated, since it is not likely to occur *in vivo* (Greenbaum et al., 1971).

From the above discussion (and Table 1) one can conclude that the use of the double substrate procedure of Glock and McLean (1953) probably underestimates G6PD activity, and, therefore, the simpler, single substrate procedure is likely to be the better one to use.

APPENDIX III. Double-Reciprocal Plots Showing the Effects of Different Substrate Concentrations and Assay Temperatures on the Activity of G6PD and 6PGD

Figure 1. Double-reciprocal plot showing the effect of different substrate (G6P) concentrations on the activity of white sucker (*Catostomus*) liver G6PD at different assay temperatures. Saturating concentrations of NADP$^+$ were used. The data shown were obtained using the liver homogenate from winter caught fish, except for the assay at 0.0 C which used homogenate from May caught fish. Km and Vmax values are presented in text Tables 1 and 5, respectively. For each equation presented below, n = 1.

Regression equations are:

(Winter fish)

(22.0 C) 1/velocity = 0.6886 + 0.0858 (1/mM G6P)

(12.0 C) " = 1.5026 + 0.1202 (")

(4.5 C) " = 2.9443 + 0.2219 (")

(2.3 C) " = 4.5317 + 0.2238 (")

(May fish)

(0.0 C) " = 4.9239 + 0.4065 (")

(Summer fish)

(23.0 C) " = 0.6731 + 0.0836 (")

(13.5 C) " = 1.5871 + 0.1267 (")

(5.7 C) " = 3.0705 + 0.2223 (")

Velocity x 10^4 = nmol NADPH produced/min/gram wet weight

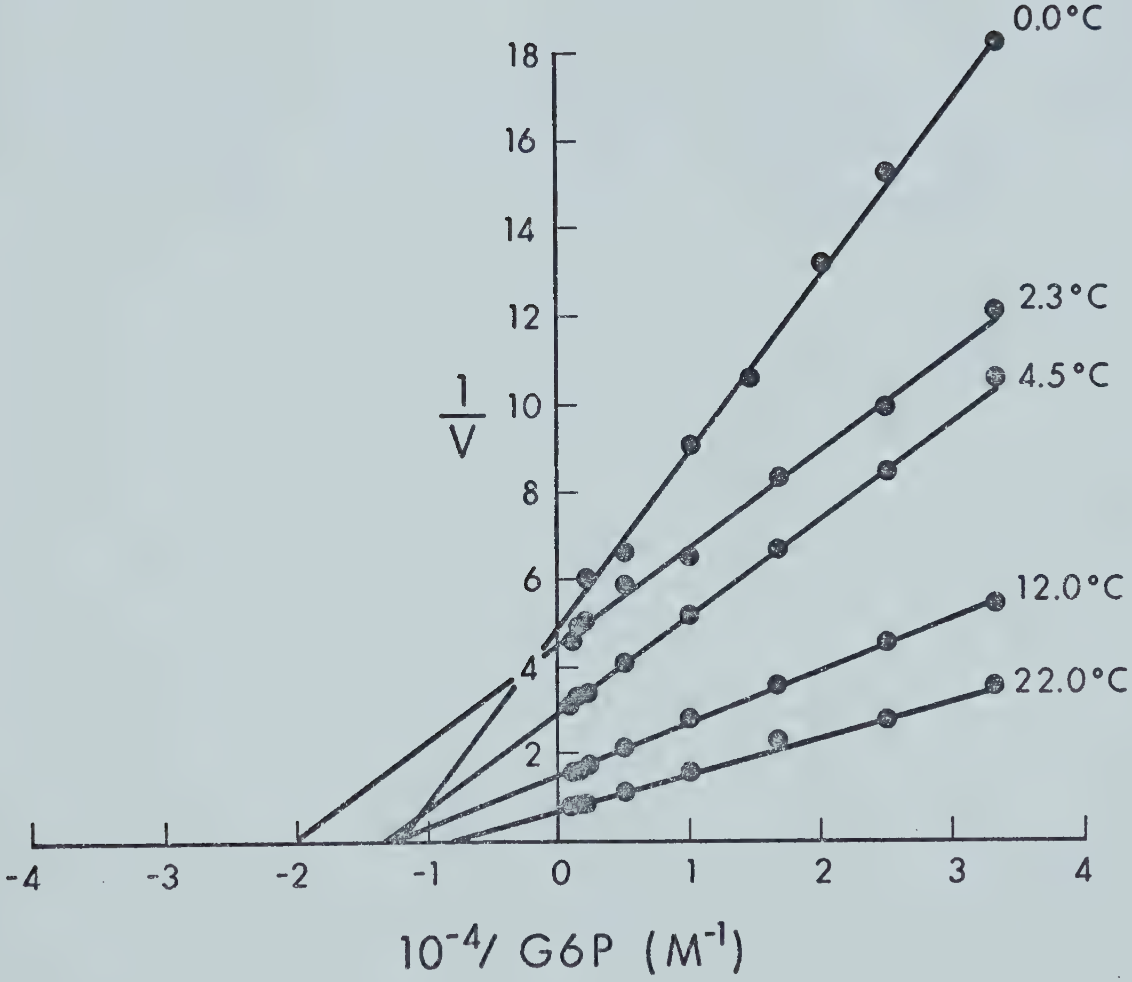

0.0°C
2.3°C
4.5°C
12.0°C
22.0°C
18
16
14
12
10
8
6
4
2
$\frac{1}{V}$
-4
-3
-2
-1
0
1
2
3
4
10^{-4}/ G6P (M^{-1})

Figure 2. Double-reciprocal plot showing the effect of different substrate (G6P) concentrations on the activity of northern pike (*Esox*) liver G6PD at different assay temperatures. The data shown were obtained using the liver homogenate from winter caught fish. Other details as in Figure 1.

Regression equations are:

(Winter fish)

(22.2 C) 1/velocity = 9.7600 + 0.5759 (1/mM G6P)

(11.3 C) " = 24.6898 + 1.0770 (")

(5.0 C) " = 70.0173 + 2.9244 (")

(1.8 C) " = 129.5898 + 5.4617 (")

(0.6 C) " = 151.6137 + 8.7038 (")

(Summer fish)

(22.0 C) " = 12.0920 + 0.7290 (")

(13.0 C) " = 28.3915 + 1.2414 (")

(5.5 C) " = 102.2348 + 4.1274 (")

Velocity x 10^4 = nmol NADPH produced/min/gram wet weight

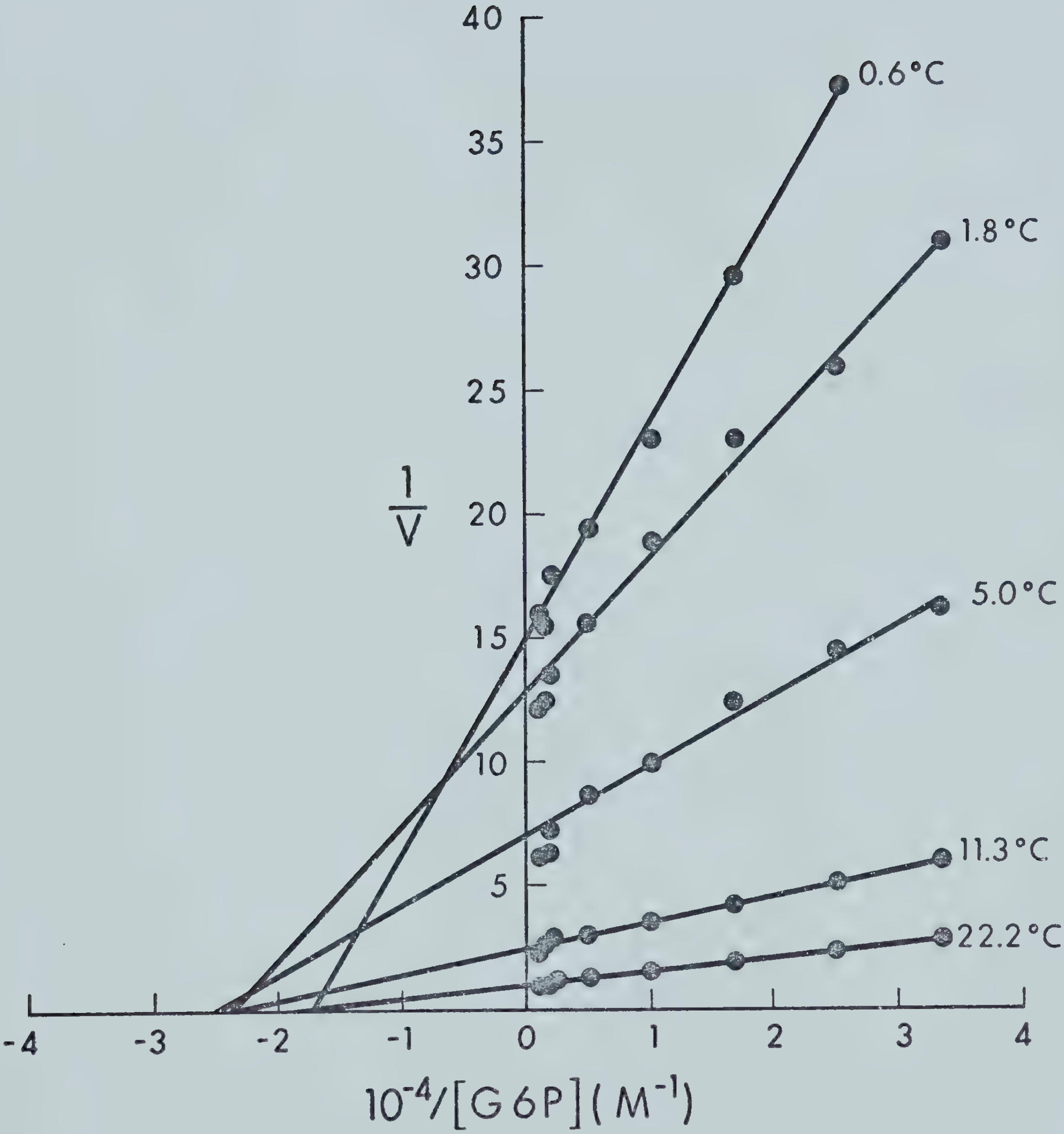

40
35
30
25
20
15
10
5
1/V
0.6°C
1.8°C
5.0°C
11.3°C
22.2°C
-4
-3
-2
-1
0
1
2
3
4
10⁻⁴/[G6P](M⁻¹)

Figure 3. Double-reciprocal plot showing the effect of different substrate (G6P) concentrations on the activity of goldfish (*Carassius*) liver G6PD at different assay temperatures. The liver homogenate was from cold-acclimated fish. At each assay temperature, n = 1. Because of the quantitative variability among different goldfish homogenates (text Table 5), regression equations are not shown, and the velocity is expressed as Δ Absorbance/min/0.2 ml of a 3% homogenate. Other details as in Figure 1.

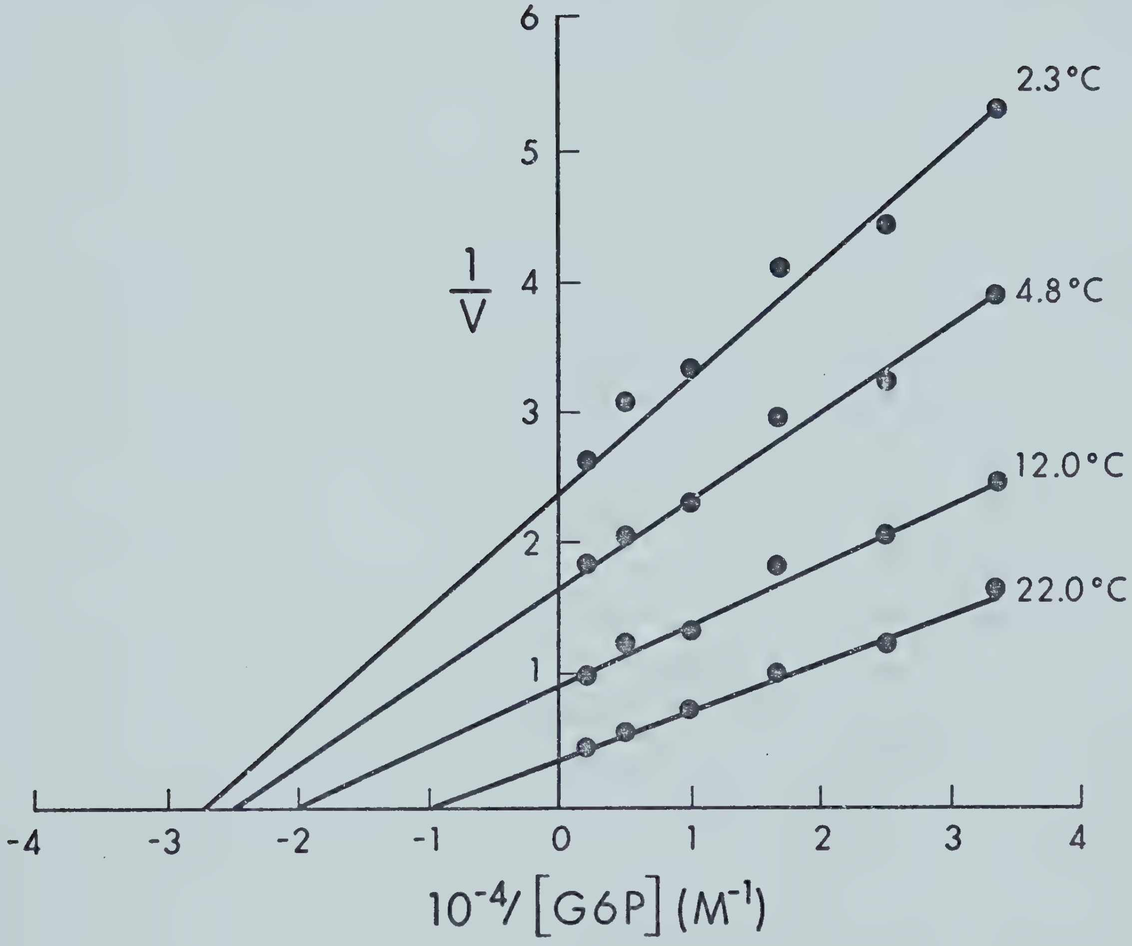

6
5
4
3
2
1
$\frac{1}{V}$
2.3°C
4.8°C
12.0°C
22.0°C
-4
-3
-2
-1
0
1
2
3
4
$10^{-4}/[G6P]\ (M^{-1})$

Figure 4. Double-reciprocal plot showing the effect of different substrate (6PG) concentrations on the activity of white sucker (*Catostomus*) liver 6PGD at different assay temperatures. Saturating concentrations of $NADP^+$ were used. The liver homogenate was from May caught fish. Km and Vmax values are presented in text Figure 4B and text Table 6, respectively. For each equation presented below, n = 1.

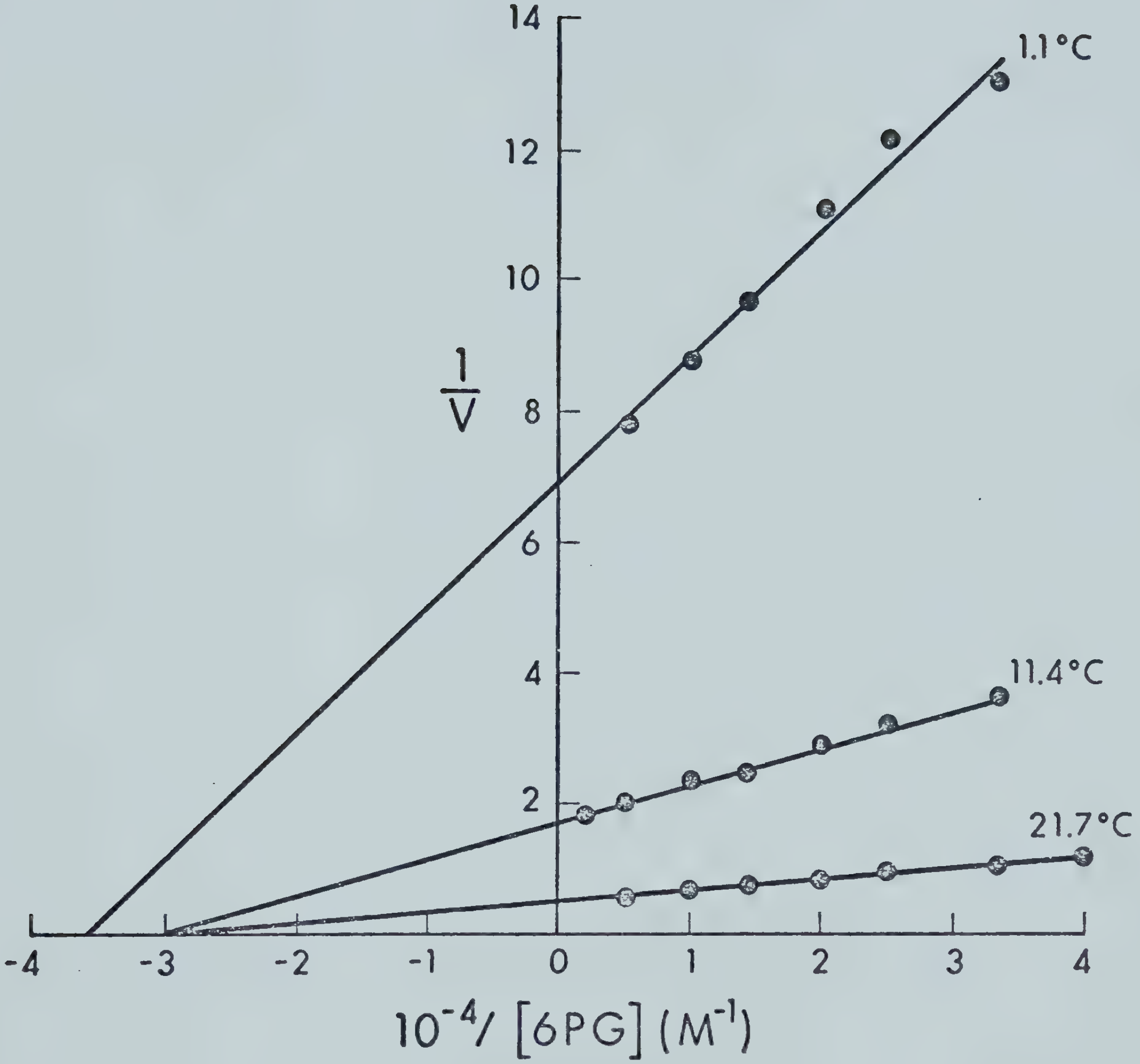
Regression equations are:

(21.7 C) 1/velocity = 4.9754 + 0.1806 (1/mM 6PG)

(11.4 C) " = 17.2365 + 0.5901 (")

(1.1 C) " = 69.7866 + 1.9409 (")

Velocity x 10^4 = nmol NADPH produced/min/gram wet weight


14
12
10
8
6
4
2
1/V
1.1°C
11.4°C
21.7°C
-4
-3
-2
-1
0
1
2
3
4
10⁻⁴/ [6PG] (M⁻¹)

APPENDIX III (Continued)

Figure 5. Double-reciprocal plot showing the effect of different substrate (6PG) concentrations on the activity of northern pike (*Esox*) liver 6PGD at different assay temperatures. The liver homogenate was from summer caught fish. Other details as in Figure 4.

Regression equations are:

(21.3 C) 1/velocity = 10.5062 + 0.4896 (1/mM 6PG)

(11.1 C) " = 34.6179 + 1.4326 (")

(1.0 C) " = 134.0476 + 5.3535 (")

Velocity x 10^4 = nmol NADPH produced/min/gram wet weight

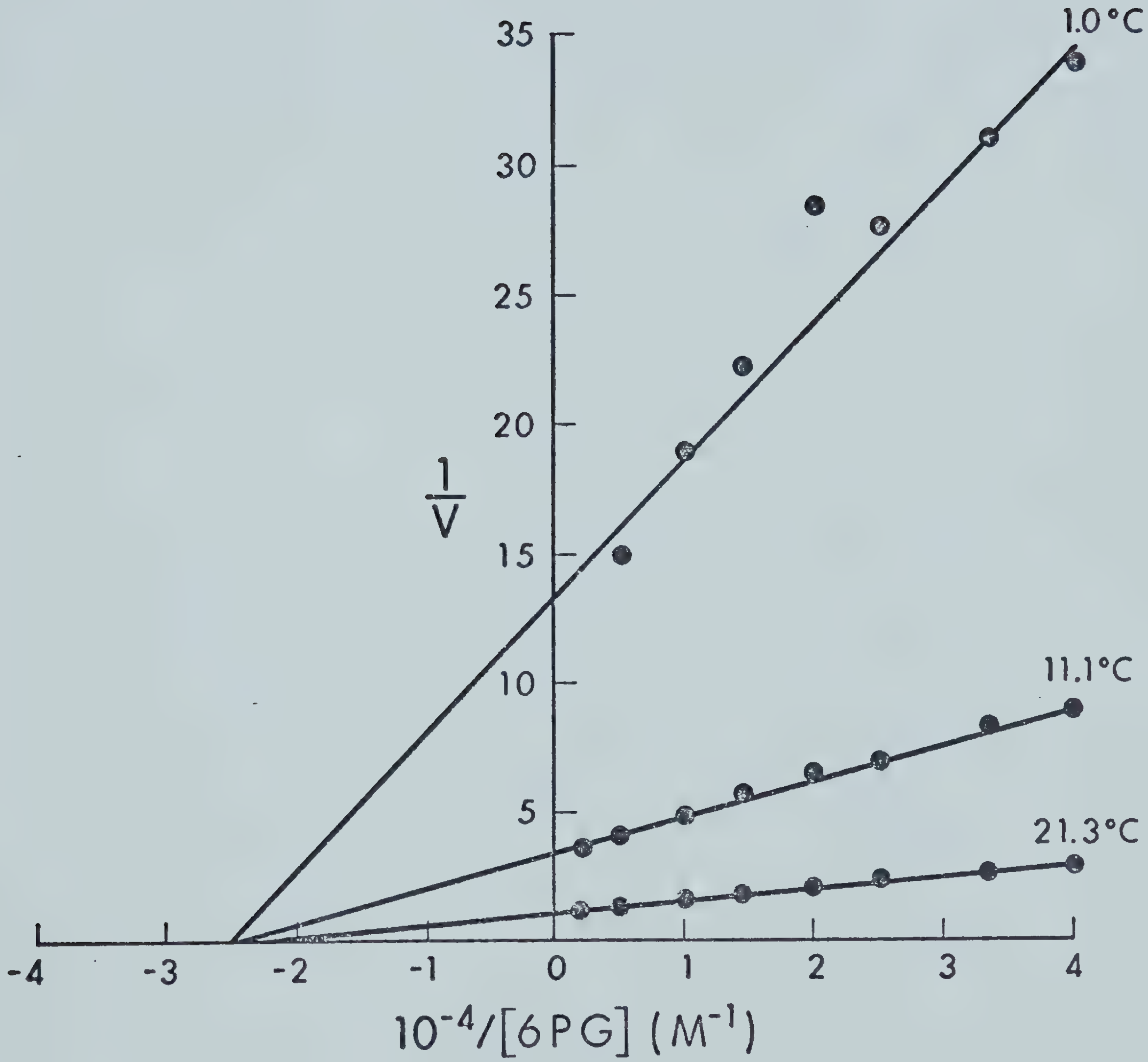
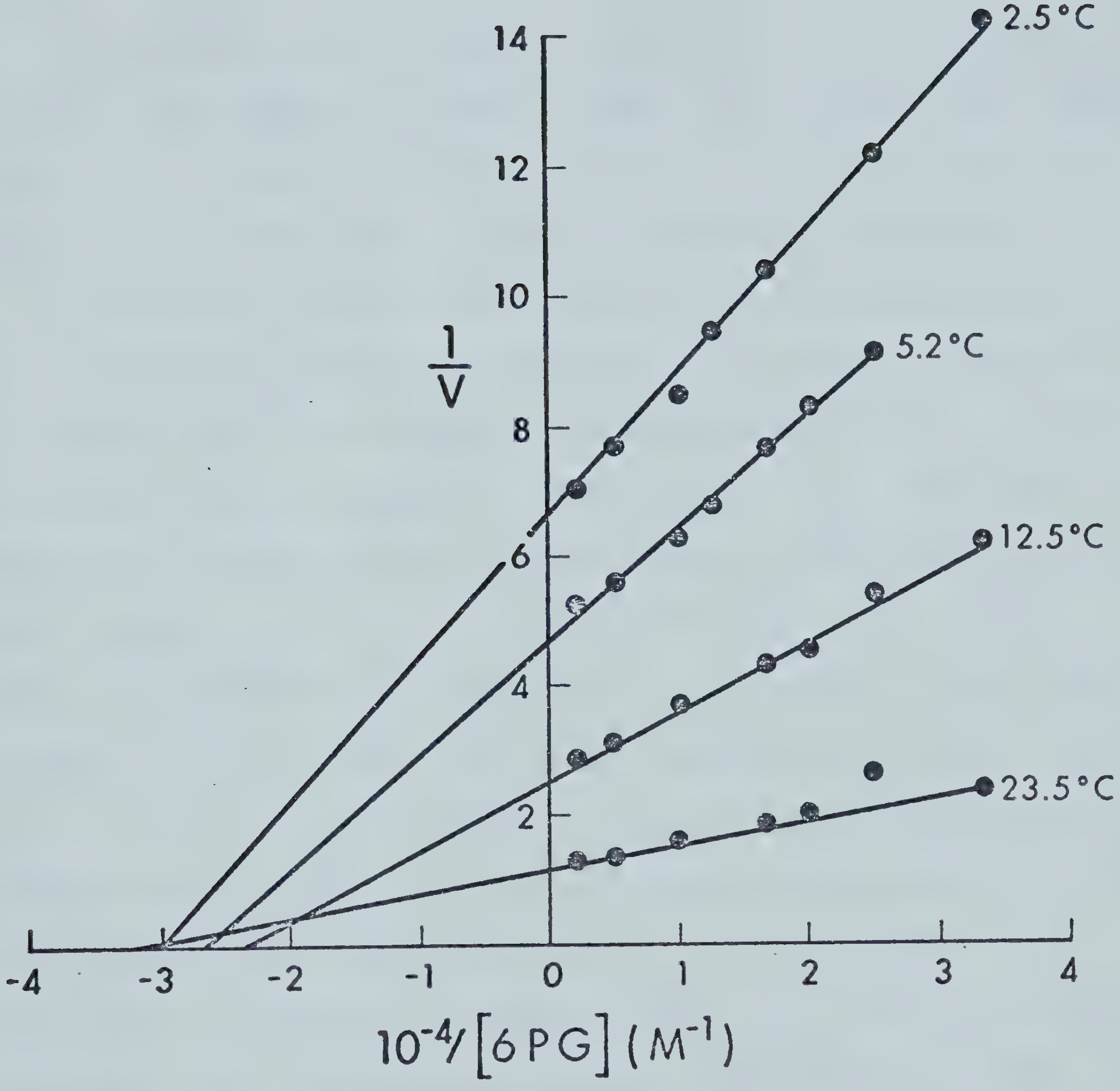

1.0 °C
11.1°C
21.3°C
1/V
35
30
25
20
15
10
5
-4
-3
-2
-1
0
1
2
3
4
10^{-4}/[6PG] (M^{-1})

Figure 6. Double-reciprocal plot showing the effect of different substrate (6PG) concentrations on the activity of goldfish (*Carassius*) liver 6PGD at different assay temperatures. Saturating concentrations of $NADP^+$ were used. The liver homogenate was from warm-acclimated fish. At each assay temperature, $n = 1$. Because of the quantitative variability among different goldfish homogenates (text Table 6), regression equations are not shown, and the velocity is expressed as Δ Absorbance/min/0.2 ml of a 5% homogenate. Km and Vmax values for goldfish 6PGD from different homogenates are presented in text Tables 3 and 6, respectively.


2.5 °C
5.2 °C
12.5 °C
23.5 °C
$\frac{1}{V}$
14
12
10
8
6
4
2
-4
-3
-2
-1
0
1
2
3
4
$10^{-4}/[6PG]\ (M^{-1})$

APPENDIX IV. Inhibition of Glucose-6-Phosphate Dehydrogenase

Of the potential physiological inhibitors examined (see below), the most potent inhibitor of G6PD was NADPH. The Ki(NADPH) values (text Table 2) were determined from Dixon plots (Figs. 1A, B, and 2) and are discussed in the text (Results, section C; Discussion, section C).

It has been suggested that other organic molecules may control the G6PD reaction, particularly ATP (Avigad, 1968; Yoshida, 1973) and NADH (Sanwal, 1970). One would not expect inhibition of G6PD or 6PGD by ATP since the pentose phosphate pathway should be most active when cellular ATP concentrations are high and energy is being stored in the form of lipids. At low ATP concentrations (<0.4 mM), there was no inhibition of goldfish liver G6PD (Fig. 3). It appears, in fact, that there may have been a slight activation. Hochachka et al. (1970) also found activation of pentose phosphate pathway activity by ATP at low concentrations (<0.5 mM) in the king crab (*Paralithodes camtchatica*) gill. There appears to be no other report of this in the literature. The concentration of ATP found in mammalian whole liver tissue preparations is at least 2 mM (2 µmoles/gram wet weight, Greenbaum et al., 1971). At this concentration of ATP, goldfish liver G6PD was inhibited by only 10% (Fig. 3) and pentose phosphate pathway activity in the king crab gill by about 20%. Although the actual concentration of ATP in the cytoplasm is not known (Newsholme and Start, 1973), the relatively low *in vitro* inhibition of goldfish liver G6PD by ATP concentrations found in whole tissue suggests that ATP is not a major regulator of G6PD activity *in vivo*.

Figure 1A (Upper). Dixon plots to determine the inhibitor constant, Ki(NADPH), for white sucker (*Catostomus*) liver G6PD. The horizontal lines intersect the y-axis (1/v axis) at 1/Vmax for the Km($NADP^+$) (text Fig. 2A) at their respective temperatures. G6P concentration was 0.2 mM; $NADP^+$ concentration was 0.05 mM. NADPH concentrations were varied. The regression equations are:

(22.0 C) $1/v = 2.1960 + 0.2192$ (mM NADPH x 10^2)

(2.0 C) $1/v = 3.2869 + 0.2544$ (mM NADPH x 10^2)

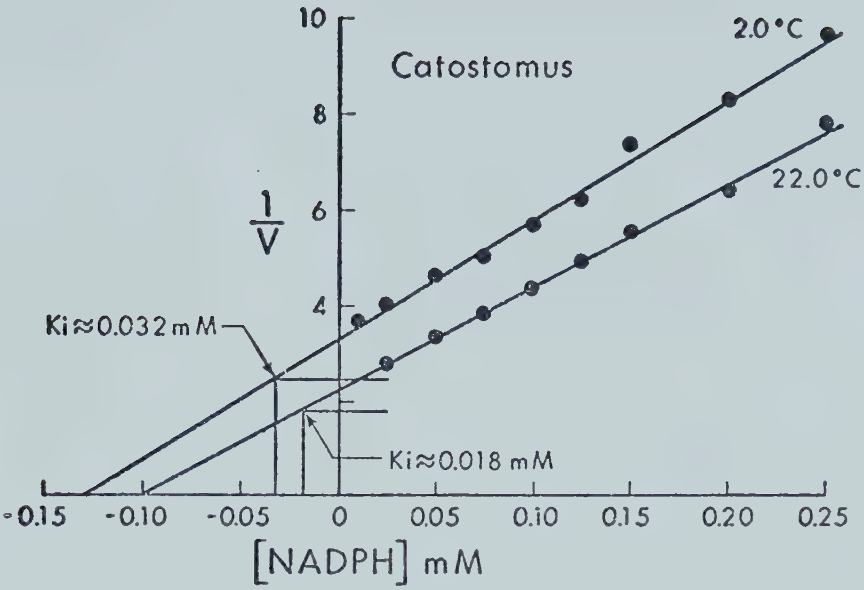
Velocity = Δ Absorbance/min

Figure 1B (Lower). Dixon plot to determine the inhibitor constant, Ki(NADPH), for northern pike (*Esox*) liver G6PD, as described in Figure 1A. Km($NADP^+$) is shown in text Figure 2B.

(22.0 C) $1/v = 3.9814 + 0.3364$ (mM NADPH x 10^2)

(2.0 C) $1/v = 10.2814 + 0.7415$ (mM NADPH x 10^2)

Velocity = Δ Absorbance/min


Catostomus
2.0°C
22.0°C
Ki≈0.032 mM
Ki≈0.018 mM
1/V
10
8
6
4
-0.15
-0.10
-0.05
0
0.05
0.10
0.15
0.20
0.25
[NADPH] mM

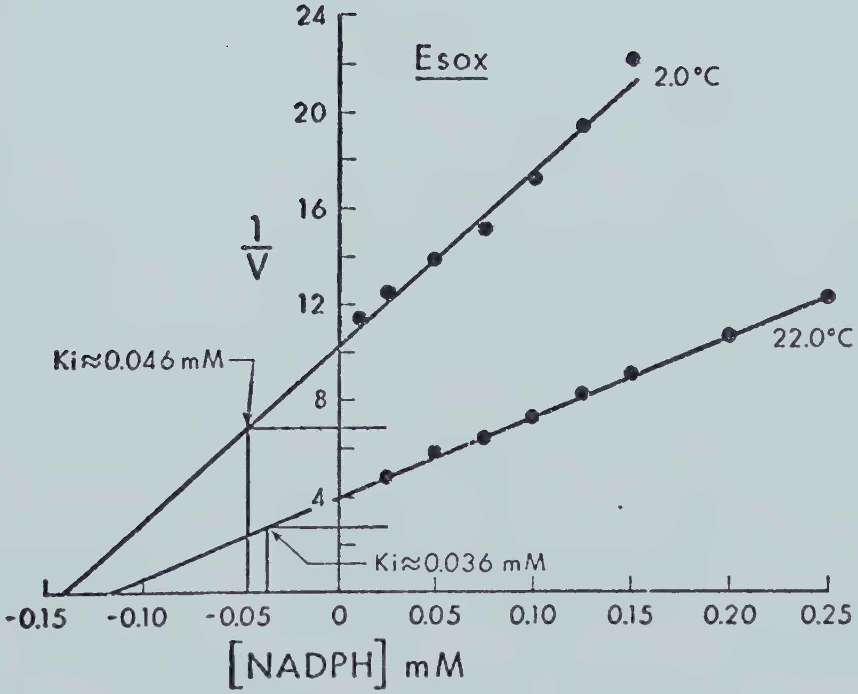

Esox
2.0°C
22.0°C
Ki≈0.046 mM
Ki≈0.036 mM
1/V
24
20
16
12
8
4
-0.15
-0.10
-0.05
0
0.05
0.10
0.15
0.20
0.25
[NADPH] mM

Figure 2. Dixon plot to determine the inhibitor constant, Ki(NADPH), for goldfish (*Carassius*) liver G6PD at 22.0 C. The $NADP^+$ concentration was 0.14 mM. NADPH concentrations were varied.

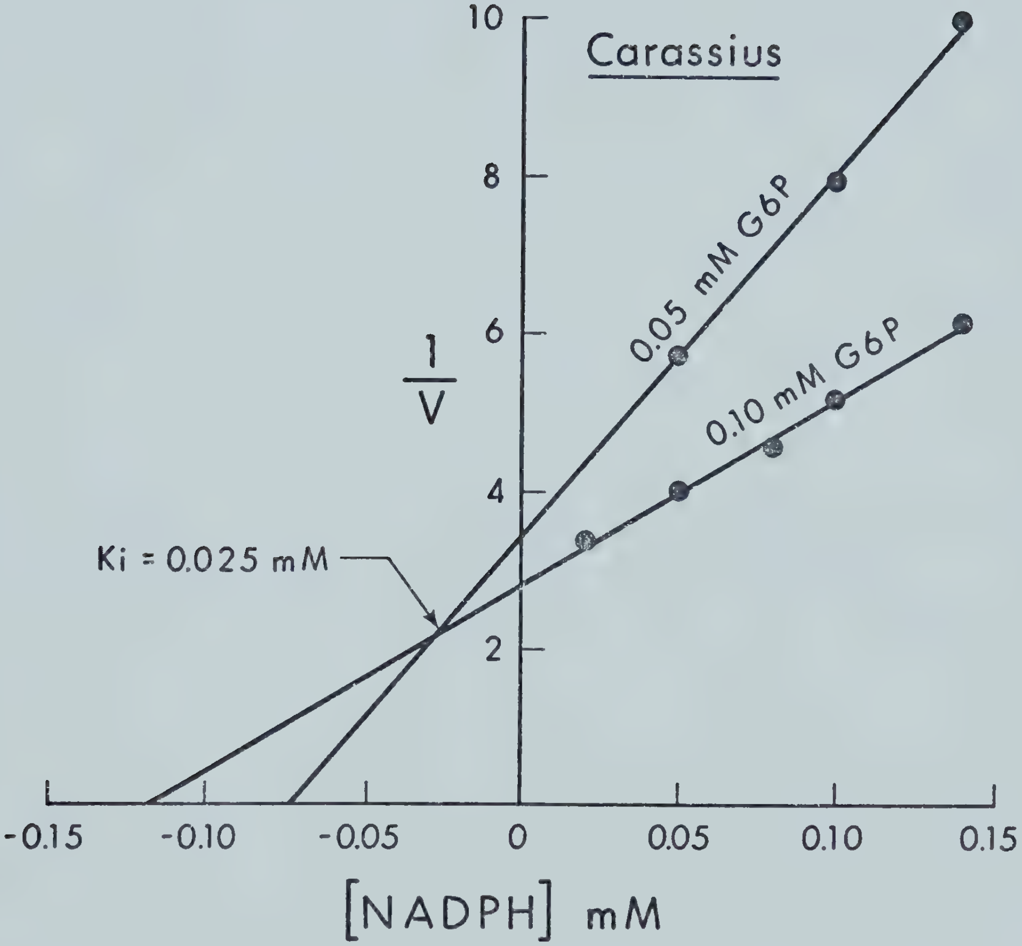
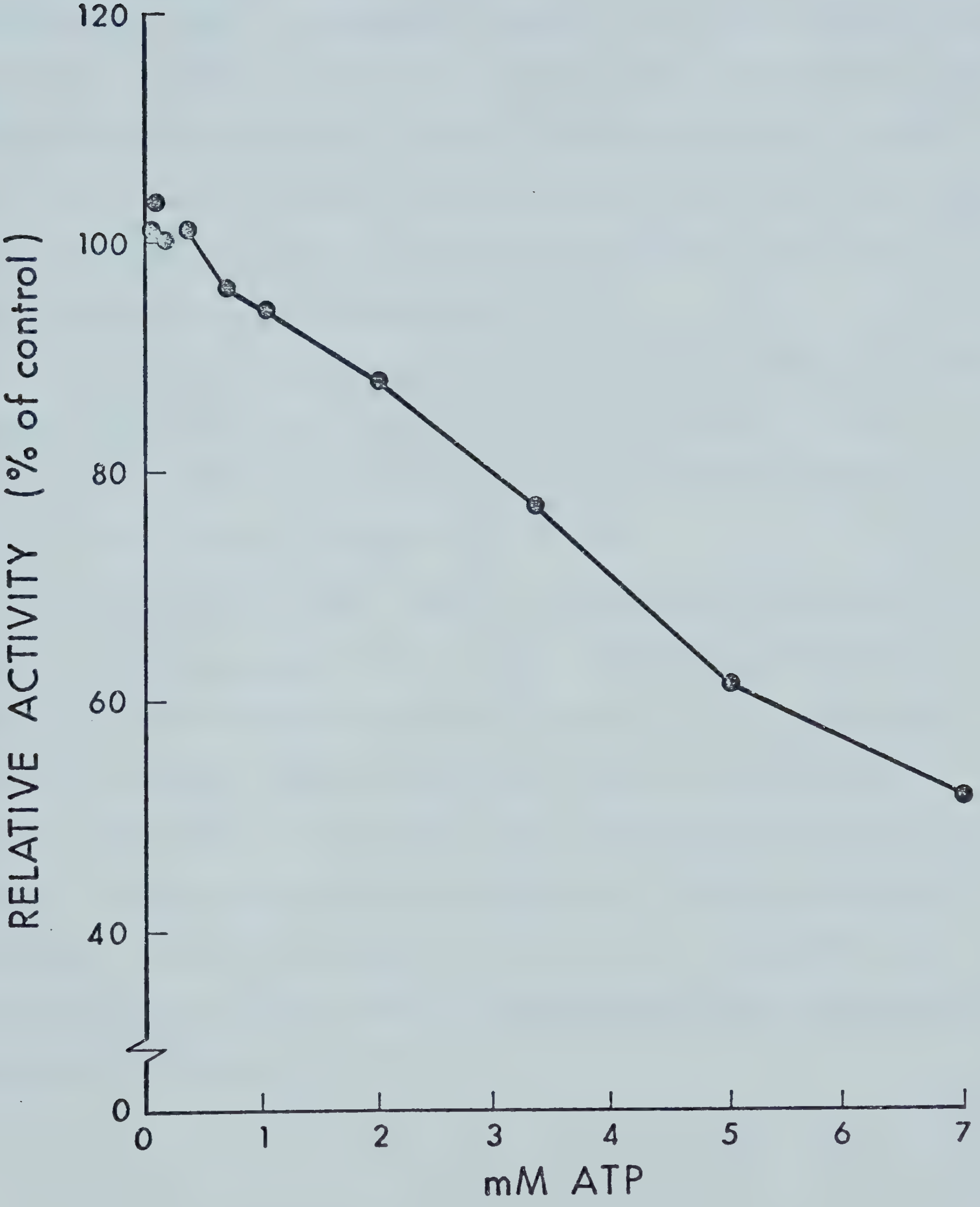

Carassius
0.05 mM G6P
0.10 mM G6P
Ki = 0.025 mM
1/V
10
8
6
4
2
-0.15
-0.10
-0.05
0
0.05
0.10
0.15
[NADPH] mM

Figure 3. The effect of ATP on goldfish (*Carassius*) liver G6PD activity. The G6P concentration was 0.05 mM; $NADP^+$ was saturating; and the concentration of $MgCl_2$ was 2.0 mM. The control cuvette contained no ATP. The assay temperature was 22.3 C.


RELATIVE ACTIVITY (% of control)
120
100
80
60
40
0
0
1
2
3
4
5
6
7
mM ATP

NADH regulates a number of reactions in the cell. Sanwal (1970) has shown that NADH is an allosteric regulator of *E. coli* G6PD and effects the binding of $NADP^+$, and not G6P. In the present study (Table 1), NADH inhibited goldfish liver G6PD, but much less severely than in Sanwal's (1970) study, and at inhibitor concentrations (0.3 mM) higher than those found in the cell (on the basis of calculated NAD^+/NADH ratios from Krebs and Veech, 1969; Greenbaum et al., 1971).

Citrate, even at high concentrations, did not inhibit or activate goldfish liver G6PD activity (Table 1), which is contrary to the inhibition of carbon flow through the king crab pentose phosphate pathway by citrate found by Hochachka et al. (1970).

In the above inhibition studies, as elsewhere in this work, a number of different homogenates were used. Despite the quantitative variability of the homogenates (see text Table 8), it is assumed (due to identical Km(G6P) values for G6PD from warm- and cold-acclimated goldfish, text Table 1, for example), that the enzymes from all homogenates of a particular species and organ exhibit the same qualitative properties. Hence, it is assumed in this work that homogenates from different groups of fish may be used interchangeably when studying the qualitative properties of G6PD.

APPENDIX IV (Continued)

Table 1. The effect of citrate and NADH on goldfish (*Carassius*) liver G6PD activity. Final concentrations in the assay medium are shown. The G6P concentration was 0.2 mM; $NADP^+$ was saturating. The assay temperature was 22 C.

	Concentration (mM)	% Relative activity
Citrate	0.0	100
"	0.1	100
"	1.0	100
NADH	0.0	100
"	0.2	86
"	0.3	70
"	0.3 + 0.8 NAD^+	70

APPENDIX V

Table 1. Measurements of *in vivo* G6P and 6PG concentrations. When the intracellular water content, and the extracellular volume of a tissue are considered, the actual *in vivo* intracellular substrate concentrations can be calculated, and are generally more than double the concentrations given below (Newsholme and Start, p. 31, 1973). Therefore, the values given below should be considered, at best, only minimum estimates of *in vivo* intracellular G6P and 6PG concentrations.

Animal and tissue	Substrate	Concentration nmoles/gram (wet weight)	Conditions	Reference
Goldfish muscle	G6P	202	cold-acclimated	1
		837	warm-acclimated	
Blowfly flight muscle (*Phormia regina*)	G6P	175	--	2
Rat liver	G6P	217	control	3
	6PG	14	"	
	G6P	86	starved	3
	6PG	14	"	
	G6P	120	lipogenic	3
	6PG	56	"	
Rat liver	G6P	169	starved	4
	6PG	31	"	
	G6P	187	lipogenic	4
	6PG	44	"	
Rat liver	6PG	27	--	5

References:

1. Freed (1971).
2. Sacktor and Wormser-Shavit (1966).
3. Greenbaum et al. (1971).
4. Sapag-Hagar et al. (1973).
5. Arese (1964).

APPENDIX VI. Lipid and Glycogen

A. Materials and Methods

i. Lipid determinations. Samples of liver and muscle from goldfish and suckers, as well as whole goldfish, were cut up into small pieces and lyophilized. When dry, the samples were ground with a mortar and pestle, and either analysed directly, or, in the case of whole goldfish, ground to 20-mesh size in a Wiley Mill (A. H. Thomas Co., Phila.). Later in the study, a Krups KM 75 electric coffee mill (Krups, West Germany) was used for homogenizing chunks of the lyophilized tissue. With this equipment, the degree of homogenization was roughly equivalent to that from the Wiley Mill, but the recovery of material was greater and the time required per sample was considerably less. Total lipid in 0.1-0.7 g portions of the dried, homogenized tissue was determined gravimetrically after extraction for five hours with chloroform:methanol (2:1) in a Soxhlet extraction apparatus, followed by an aqueous calcium chloride (0.05%) wash of the extract, as described by Folch et al. (1957). Duplicate samples were used in the analysis of goldfish tissue, but only single determinations were made in the analysis of sucker tissue.

Preliminary tests and development of optimal procedures for the chloroform:methanol extraction in this laboratory are described by Medford (1976).

ii. Glycogen determinations. Cold- and warm-acclimated goldfish were taken from their tanks, and liver and muscle samples were removed within one minute. The samples were placed in liquid nitrogen (-196 C)

within two minutes of the animals' removal from the water, and stored in the liquid nitrogen or on dry ice (-76 C) until analysed.

The procedure used to determine glycogen was essentially the microdetermination of Seifer et al. (1949) with some of the modifications of van Handel (1965).

Tissues were minced and 50 to 300 mg samples were boiled in 1 ml of 30% KOH for 20 minutes. Saturated sodium sulfate (0.05 ml) and 95% ethanol (0.5 ml) were then added to duplicate 0.4 ml aliquots of the extract, which were then gently reheated, cooled, and the precipitate spun down (3,000 x g at 0 C for 45 minutes in a RC-2 refrigerated centrifuge). The tubes were then drained and the precipitate (glycogen) resuspended in 5 ml of water. Proper dilutions were made, anthrone reagent (0.2% anthrone in 95% sulfuric acid) added, and the color allowed to develop for 15 minutes in an 85 C water bath. The tubes were cooled and the absorbance was measured at 620 nm 22 to 25 minutes after the addition of anthrone. No change in color occurred between 20 and 30 minutes after anthrone was added.

Each day tissues were analysed, duplicate standard glucose solutions were analysed in the same manner. Linearity was observed between glucose concentrations of 10 to 75 μg/liter. Glycogen-glucose values were calculated from the equation obtained by least squares linear regression of the standard glucose solution values. No standard curve had a correlation coefficient of less than 0.9995.

Glycogen analysis of sucker and pike tissues was not attempted because of the erroneous values one would obtain due to the glycogenolysis and glycolysis which would occur as the fish struggled in the net and were transported back to the laboratory (Manohar, 1970).

B. Results and Discussion

There was no significant difference ($p > 0.1$) in the whole-fish lipid content of cold- and warm-acclimated goldfish (Table 1). Both groups were taken from the same shipment of fish. One group was kept at room temperature, while the other group was cold-acclimated (2 C)(and fed) for two months (see protocol in Materials and Methods). Because of the significant positive correlation (0.65, personal observation) between length (presumably weight as well) and lipid content, the cold- and warm-acclimated groups were carefully chosen to be virtually identical in length, total weight, and weight/length ratio.

Unlike whole-fish lipid, glycogen content in goldfish muscle tissue showed a significant ($p < 0.001$) increase in cold-acclimated fish (Table 1). No acclimation-dependent comparisons of liver glycogen content were made in this work. The liver glycogen content found in goldfish (Table 1) was similar to that found by Johnston and Goldspink (1973) in the liver of crucian carp.

The liver of summer-caught suckers contains about four times more lipid than the muscle tissue (Table 1), when expressed as percentage composition. The muscle tissue, however, comprises perhaps 45-50% of the body weight (personal observation), while the liver is approximately 2% of the total body weight (personal observation). A comparison of the estimated total amounts of lipid each tissue contained is shown in Table 2, along with data on northern pike from Medford (1976).

APPENDIX VI (Continued)

Table 1. Lipid and glycogen content of some tissues from suckers (*Catostomus*) and goldfish (*Carassius*); mean ± SEM (n). Student's t-test for unpaired samples was used to determine whether the differences between groups were significant ($p < 0.05$).

Species	Acclimation or season	Tissue	Lipid[1]	Glycogen[2]
Carassius	cold-acclimated	whole fish	24.5±2.6 (14)[a]	-----
"	warm-acclimated	whole fish	21.0±2.4 (16)[a]	-----
Catostomus	summer (July, Aug.)	liver	24.5±2.4 (6)	-----
Catostomus	summer (July, Aug.)	muscle	6.1±0.4 (9)	-----
Carassius	cold-acclimated	liver	-----	22.0 ±1.5 (6)
Carassius	cold-acclimated	muscle	-----	0.60±0.05 (12)[b]
"	warm-acclimated	muscle	-----	0.18±0.01 (6)[b]

[1] % dry weight.

[2] % wet weight.

[a] Not significant $p > 0.1$.

[b] Significant difference $p < 0.001$.

APPENDIX VI (Continued)

Table 2. Estimates of the total lipid content of the liver and muscle of white suckers (*Catostomus*) and northern pike (*Esox*). White sucker values are from Table 1. Northern pike values are recalculated from the lipid contents found by Medford (1976). The liver weight is approximately 2% of the total body weight, and the muscle is about 45 to 50% of the total body weight (personal observations). It is likely that the total lipid content of the body will be somewhat greater than double the total lipid for liver and muscle alone, since other tissues, such as the brain and gonads, probably contain considerable amounts of lipid.

		Catostomus		*Esox*	
		July	March	July	March
LIVER	% wet wt	6.0	---	7.2	3.4
	total mg	1,200		910 (13 g)	813 (23.9 g)
MUSCLE	% wet wt	1.3	---	0.63	0.72
	total mg (450 g)	5,850		2,835	3,240
TOTAL	(Liver, muscle)	7,050	---	3,750	4,050

APPENDIX VII. Experimental Protocol, Calculation of Activity, Km, and Vmax.

Enzyme activity in the homogenate was assayed by recording the reduction of $NADP^+$ as shown by the increase in absorbance at 340 nm. The actual spectrophotometer readings during several assays are shown in Figure 1. The procedure routinely followed was to add $NADP^+$ last to the assay mixture in the cuvette, then record the increase in absorbance (Assay A, Fig. 1). A constant rate of increase in absorbance was measured for a minimum of two minutes (usually 3 to 5 minutes), and this was considered to be the initial velocity, expressed as Δ absorbance per minute. The increase in absorbance with time was proportional to the concentration of tissue homogenate (Fig. 2) and, presumably, to the enzyme concentration. Generally, homogenates from only one group of fish were tested for proportionality of activity for each species and enzyme, and usually only over a two-fold concentration range.

The homogenate used in the assays contained not only enzymes found in the cytoplasm of the cell, but also enzymes from cell organelles which were lysed during the homogenization procedure (see Materials and Methods). However, in the present study no data are available, for instance, on the possible denaturation and consequent lowered activity of the dehydrogenases due to catheptic enzymes from ruptured lysosomes, or on possible pH changes which might cause precipitation of enzyme proteins during homogenization or storage. However, homogenates were normally used within one month of preparation, and no

Figure 1. Time courses of typical dehydrogenase assays. The liver homogenates in Assays A, B, D were from summer-caught northern pike (*Esox lucius*). Assays A and B are from the same homogenate. In Assay C, the liver homogenate was from warm-acclimated goldfish (*Carassius auratus*) and was freshly prepared, not previously frozen as were the pike homogenates. Assay temperature was 22 C. All concentrations given below are final concentrations in the assay mixture. For additional components of the assay mixture, see Materials and Methods. Each assay represents raw data from a single determination.

Assay A (G6PD). Assay mixture contained 0.2 ml of 20% liver homogenate; 0.1 mM G6P; and 0.4 mM $NADP^+$. The $NADP^+$ was added last (arrow at 0 time). Δ absorbance = 0.043/minute.

Assay B (G6PD). Assay mixture contained 0.1 ml of 20% liver homogenate; 0.4 mM $NADP^+$; and 0.1 mM G6P. The G6P was added last (arrow between 3 and 4 minutes). Δ absorbance = 0.022/minute.

Assay C (6PGD). Assay mixture contained 0.025 ml of 20% goldfish liver homogenate; 0.3 mM $NADP^+$; and 0.1 mM 6PG. The 6PG was added last (arrow between 4 and 5 minutes). Δ absorbance = 0.050/minute.

Assay D Cuvette contained 0.2 ml of 25% liver homogenate; 0.2 mM G6P; and 0.05 mM NADPH. This cuvette was used as a "blank" (during inhibition studies, Appendix IV), and so $NADP^+$ was not added. Absorbance decreased by less than 0.002/minute.

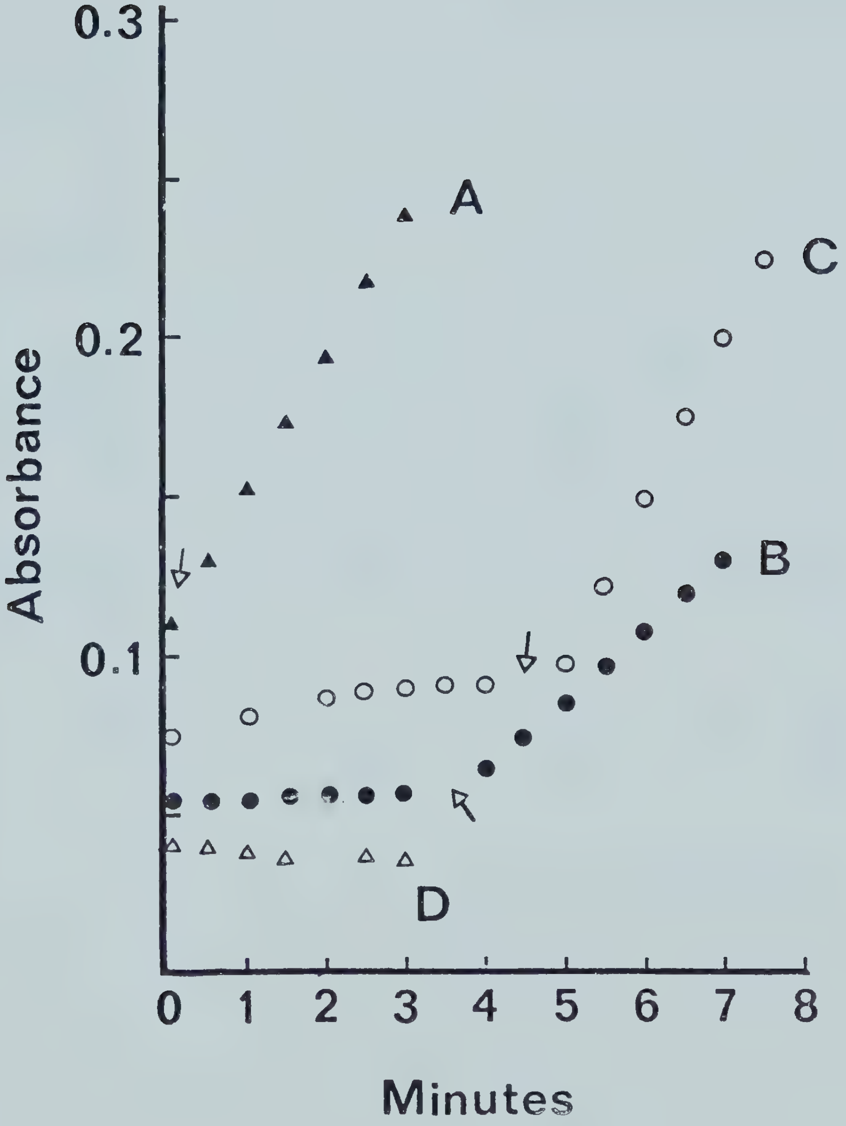
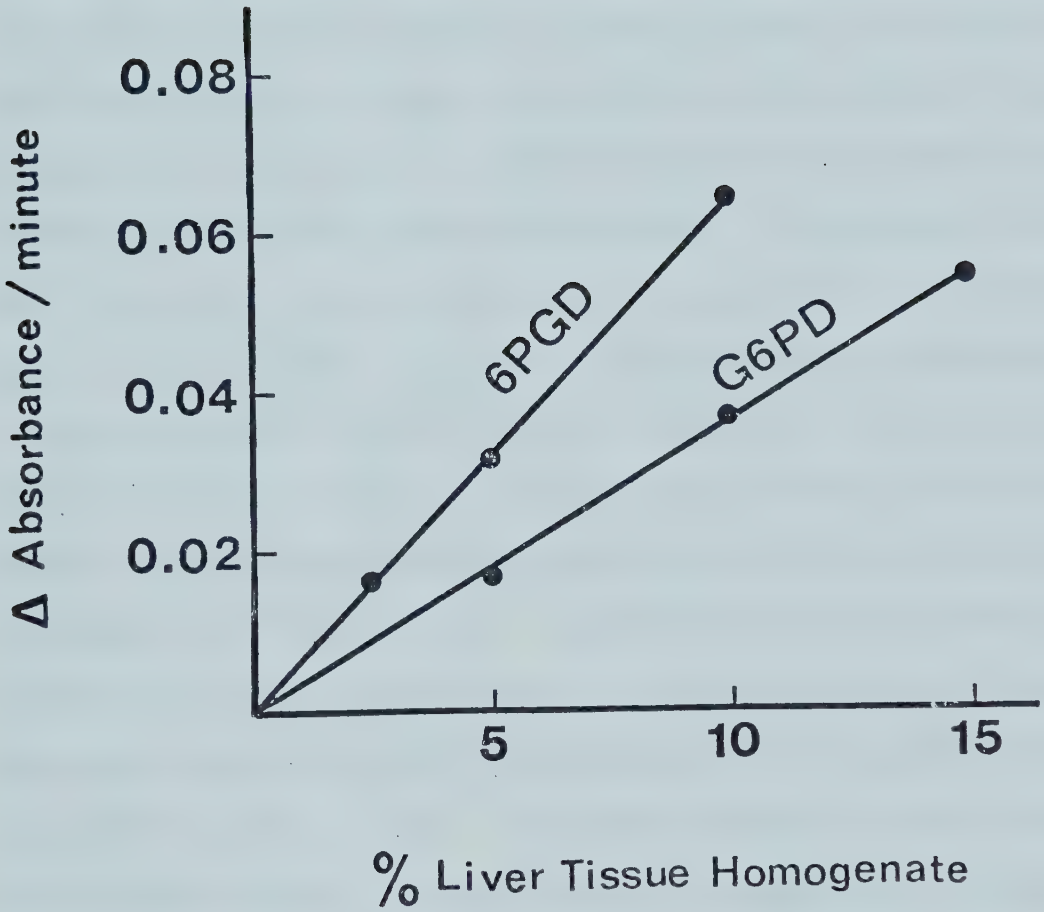

0.3
0.2
0.1
Absorbance
0
1
2
3
4
5
6
7
8
Minutes
A
B
C
D

Figure 2. The effect of different homogenate concentrations on pike (*Esox lucius*) liver G6PD and 6PGD activity. The G6PD assay mixture contained (final conc.) 0.5 mM G6P and 0.4 mM NADP$^+$ (added last). The 6PGD assay mixture contained (final conc.) 0.5 mM 6PG and 0.3 mM NADP$^+$ (added last). The same homogenate (0.2 ml, properly diluted), was used for both G6PD and 6PGD assays. Livers from winter-caught fish were the source of the homogenate. Assay temperature was 22 C. Each point represents a single determination.


Δ Absorbance / minute
0.08
0.06
0.04
0.02
6PGD
G6PD
5
10
15
% Liver Tissue Homogenate

detectable loss of activity occurred during this period of storage. Over one year, approximately 15-25% of the glucose-6-phosphate dehydrogenase activity was lost from the frozen liver homogenates of suckers and pike. In addition, the possibility existed that enzymes such as the various reductases (glutathione reductase, NADPH-cytochrome *c* reductase) or NADPH oxidase might be present and utilize the reduced $NADP^+$ as it was produced, thus resulting in an underestimation of the activity of the particular dehydrogenase under investigation. Alternatively, the presence of an active transhydrogenase or of activity due to endogenous substrate from the various cytoplasmic dehydrogenases not investigated in a particular assay, might increase the production of NADPH and result in an overestimation of activity for the enzyme studied in that assay (a special case, the interference of 6PGD with the G6PD assay, is discussed in Appendix II). Assay B, Fig. 1 shows that activity of $NADP^+$ reducing enzymes other than the one assayed was negligible, since no increase in absorbance occurred when $NADP^+$ was incubated with homogenate; when substrate (G6P in Assay B, Fig. 1) was added, however, a linear increase in absorbance occurred. In most assays of enzyme activity (Assays A, B, D, Fig. 1), homogenates were used which had been previously frozen. When homogenate which was freshly prepared (Assay C, Fig. 1) was assayed, however, some $NADP^+$ reduction (Δ absorbance) occurred prior to addition of substrate. This reduction was allowed to proceed until a negligible rate was recorded, usually within 3 to 5 minutes, then the assay was started by the addition of substrate (6PG in Assay C). Thus, it appears (from Assays B and C) that overestimation of activity, due to reduction of $NADP^+$ by enzymes other than the one assayed, did not occur. Incubation of

NADPH with concentrated homogenate (Assay D, Fig. 1) did not result in more than a negligible decrease in absorbance, indicating that reactions oxidizing NADPH were not active in the homogenate, and hence, did not interfere with the assay.

The activity of an enzyme at a particular substrate or coenzyme concentration was calculated from readings such as those shown in Figure 1. The change in absorbance per minute was converted to nmoles NADPH produced per minute from the equation $c = A/\varepsilon \ell$, (p. 628, Dawson et al., 1969) where:

c = concentration of product (moles/liter)
A = absorbance
ε = extinction coefficient, 6.22×10^3 liter/mole/cm
ℓ = path length of cuvette, 1 cm.

The results were corrected for the three ml volume of the assay mixture and then for either the dilution or protein content of the homogenate. The activity was expressed as nmoles NADPH produced per minute per gram of tissue or per mg protein.

The Km and Vmax values were found by conducting assays at 6 to 10 different substrate (or coenzyme) concentrations (Assay A, Fig. 1, shows one substrate concentration). The velocity (Δ absorbance/min or nmoles NADPH/min) of each assay was plotted as a reciprocal, against the reciprocal of the substrate concentration (Appendix III). The reciprocal velocity and substrate concentrations were entered into a least squares linear regression program for an Olivetti Programma 101 desk computer. Correlation coefficients for the regression equations obtained were generally higher than 0.995, and none were under 0.990. This program provided the y-intercept and slope of the line. The y-intercept equaled 1/Vmax, and the Km was found by the equation Km = Vmax x slope (p. 68, Dixon and Webb, 1964).